In vitro production of selected C-repeat binding factors (CBF) encoded by group five chromosomes in winter wheat *cv.* Norstar

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfilment of the Requirements

For the Degree of Masters of Science

In the Department of Plant Sciences

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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ABSTRACT

Winter wheat has the potential to produce 20-30% higher yields than spring wheat if it successfully overwinters. Due to early establishment in the spring, winter wheat can more efficiently utilize spring moisture, out-compete weeds, mature early and usually circumvent *Fusarium* head blight infections. However, in some regions on the Prairies production of winter wheat is constrained due to insufficient winter-hardiness in current varieties. To develop winter wheat varieties with increased cold hardiness, it is essential to gain more insight into the molecular mechanisms underlying low temperature (LT) tolerance.

Genetic analysis has revealed a major quantitative trait locus (QTL), *FrA2* on chromosome 5A that explains 50% of LT tolerance in winter wheat *cv*. Norstar. Minor QTLs for LT tolerance have also been identified on chromosomes 5B and 5D. Physical characterization of group 5 QTLs has revealed the presence of a number (≥ 60) of C-repeat binding factors (CBF), which function as transcription activators of several cold regulated genes (COR). The CBFs bind to a GC-rich region, also known as the CRT motif, present in the promoter of COR genes. Hence, determining CBF functional characteristics and their affinity towards the CRT motif would help to understand the function of each CBF isoforms and its role in LT tolerance in winter wheat. *In planta* transcription factors are present in small quantities, but are needed in relatively large quantities for functional analyses. Therefore to study the functional characteristics of CBF, they must be produced in abundance, which can be achieved by producing these proteins in recombinant form. The main objective of this work was to optimize methods to produce wheat recombinant CBF *in vitro* in quantity and quality suitable for functional studies.

Thirteen CBFs encoded by group 5 chromosomes of winter wheat were cloned from a previously constructed BAC (Bacterial Artificial Chromosome) library using DNA from a cold hardy winter wheat *cv*. Norstar. Twelve out of 13 CBF genes were inserted into protein expression vectors that were transformed into *E. coli* BLR(DE3)pLysS cells to produce TrxHisS–CBF fusion proteins. To extract recombinant CBF fusion proteins, *E. coli* cells over-expressing them were disrupted under high pressure in a cell homogenizer for 2-5 min to obtain

the TrxHisS-CBFs in cell soluble extracts. Recombinant CBFs were purified from the cell soluble extract with affinity and ion exchange chromatography procedures using an automated fast protein liquid chromatography (FPLC) system. Adequate quantities of 15 recombinant CBFs of winter wheat *cv.* Norstar were purified and selected TrxHisS-CBFs from them were assessed for functional studies.

Denaturing gel electrophoresis revealed that 11 out of 15 TrxHisS-CBFs migrated slower on SDS-PAGE which could be due to their shape, charge and presence of a large number of proline residues. Eight of the purified TrxHisS-CBFs were tested for their affinity towards the CRT motif by electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (SPR). EMSA assay showed differences in *in vitro* DNA binding ability between the recombinant CBFs encoded by group 5 chromosomes. The SPR assay suggested that compared to TrxHisS-CBFIIId-A12 (*FrA2*), TrxHisS-CBFIIId-B12 (*FrD2*) had almost eight times more affinity towards the *Wcs120* promoter CRT motif.

In conclusion, a combination of growing *E. coli* cells at low temperature (28°C), induction at low A_{600nm}, use of NPI-10 extraction buffer with mechanical disruption under high pressure by cell homogenizer resulted in all TrxHisS-CBF fusion proteins in cell soluble extracts. Affinity chromatography followed by ion exchange chromatography using a fast protein liquid chromatography (FPLC) based automated purification method proved to be optimal to purify recombinant TrxHisS-CBF fusion proteins of winter wheat *cv.* Norstar which could be suitably used for functional studies.

ACKNOWLEDGEMENTS

I sincerely thank my supervisor Prof. Ravindra N. Chibbar for his guidance, encouragement and his trust in me throughout this entire project. My sincere thanks for Dr. Monica Båga for her critical input, as well as her continuous support and guidance. Also, I would like to thank my committee members, Prof. Karen Tanino and Prof. Randy Kutcher for their suggestions and help during the study. I would like to thank Prof. Yuguang Bai, the Head of the Department of Plant Sciences and Prof. Pierre Hucl, the Graduate Chair of the Department of Plant Sciences for their help during this study.

Financial support during my studies from University of Saskatchewan, NSERC and Western Grain Research Foundation were greatly appreciated. I would like to thank my colleagues and friends in the Molecular Crop Quality Group laboratory for their support and suggestions during this study. In the end I would like to take this opportunity to thank and acknowledge my parents Mr. Sukanta Gangopadhyay and Mrs. Tripti Ganguly, whose faith in me gave me the encouragement needed to continue with my higher studies.

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LIST OF ABBREVIATIONS

ABA: Abscissic acid

ABRE: Abscissic acid binding responsive element

AP2: Apetala2

CAM: Calmodulin

CAMTA: Calmodulin binding transcription activator

CBF: C-repeat binding factor

CBL: Calcineurin β-like

CES: Combinational enhancer solution

COR: Cold regulated

CIPK: CBL interacting protein kinase

CPK: Ca^{2+} dependent protein kinase

cv.: Cultivar variety

DHN: Dehydrin

DRE: Dehydration responsive element

DREB: Dehydration responsive elements binding

DTT: Dithiothreitol

EBNA: Epstein Barr virus nuclear antigen

E. coli: *Escherichia coli*

EK/LIC: Enterokinase/ligation independent cloning

EMSA: Electrophoretic mobility shift assay

E-O: Eocene-Oligocene

FPLC: Fast Protein Liquid Chromatography

Fr: Frost resistance

FT: Flowering locus T

His-tag: Poly-histidine tag

HOS: High expression of osmotically responsive gene

ICE: Inducer of CBF expression

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IRI: Ice recrystallization inhibitor

LAC: Lactose

LB: Luria Bartonni broth

LOD: Log_{10} likelihood ratio

LT: Low temperature

LT_{50}: Median lethal temperature

Mya: Million years ago

NLS: Nuclear localizing signal

PBS: Phosphate buffer saline

PBST: Phosphate buffer saline with Tween-20

PCR: Polymerase chain reaction

PPD: Photoperiod locus

QTL: Quantitative trait locus

R:FR: Red : far red

rDNA: Nuclear ribosomal DNA genes

S-Tag: oligopeptide sequence of pancreatic RNaseA

SB: Super broth

SPR: Surface plasmon resonance

SSR: Simple sequence repeat

VRN: Vernalization

ZAT12: Zinc finger protein 12

CHAPTER 1

INTRODUCTION

1.1. The role of freezing tolerance for winter wheat production

From the equatorial hot tropical regions to the frozen Arctic tundra, the Earth's climate changes with latitude and can be divided into three main zones: the polar, the temperate and the tropical zones. Each of the zones has its own specific climate determining the development and distribution of flora and fauna. Plants being sessile organisms need to be highly adaptable to environmental conditions, and hence, have developed specific morphological, physiological and phenological traits that enable them to combat various stresses. The exposure to abiotic stresses such as extreme cold or hot temperatures or drought causes severe limitations to the growth and distribution of plant species. So, some plants have adapted to grow only during a short period of the year. Such an example is the arctic poppy (*Papaver laestadianum* Nordh.), which grows only during the short summer when days are very long. Other plants manipulate their life cycle to be independent of environmental conditions. For example, the desert sage (*Salvia eremostachya* Jeps.) does not require water at all once it establishes itself in the desert. The arctic plant *Pulsatilla* sp. Mill. grows close to the ground and is covered with thick layer of silky hairy structures to protect itself from extremely cold temperatures. At the other extreme, plants that grow in tropical climates can grow throughout the favorable year and hence, have almost no adaptation against low temperature. Temperate plants are in between these two groups and show adaptation against cold temperature.

Crop cultivation in Canada occurs within the northern temperate climate zone, primarily in the southern part bordered by the United States of America. The Prairies, where most of the Canadian crops are grown, experiences large temperature shifts during the four seasons. Survival of plants under such variable conditions requires mechanisms for protection against both high heat and excessive frost. This defense occurs naturally in native species, which acquired tolerance to temperature stresses through selection over the course of evolution. However, crop plants originating from warmer regions are not fully adapted to the Canadian climate; thus, frost in winter and extreme drought in summer are the main abiotic factors limiting winter crop production in Canada. New and better adapted varieties of crop plants have been developed

through breeding during the last century. In terms of cold-hardiness, the best Canadian winter wheat varieties were developed during the 1980s when a 30% gain in cold tolerance was obtained (Fowler, 2012). Since then, little improvement in frost-hardiness was gained in winter wheat cultivars, likely due to limited genetic variation in the germplasm used for breeding (McLeod, www.eap.mcgill.ca).

Wheat plants grown without exposure to low temperatures can only tolerate temperatures a few degrees below zero (LT_{50} ~ -3°C; Fowler *et al*., 1996). The cold-hardiness needed for winter survival is obtained upon exposure to low but above zero temperatures in combination with short day-lengths in the fall. After about six weeks of cold acclimation, the crowns which are located a few cm below the soil surface can withstand temperatures of -22°C (Fowler *et al*., 1996). It is essential that the crown is protected from frost damage as this tissue produces the new roots and shoots in the spring when plants regrow. Being cold-acclimated is not always sufficient under field conditions, especially during winters with low snow cover and air temperatures as low as -35 °C. At least five inches of snow cover is needed to ensure survival of current winter wheat cultivars on the Prairies.

In contrast to winter wheat, winter barley has a much lower ability to survive winters (LT_{50} around -10°C) whereas, winter rye cultivars (*cv.* Puma, LT_{50} = -25 to -33°C) are more winter-hardy than winter wheat (Fowler *et al*., 1996). Introgression of winter-hardiness level from rye in both winter barley and winter wheat crop improvement programs has been a goal for long. Incorporating the cold-hardiness trait of winter rye into wheat has been attempted with the production of Triticale species. However, the most cold-hardy progeny failed to show higher LT tolerance than the wheat parent. Thus, it appears, that transfer of the cold hardiness trait from rye to wheat is not a simple task (Fowler, 2012). An indication that rye-level winter survival in winter wheat is possible was provided by winter wheat cell cultures showing a very high freezing tolerance (LT_{50} = -32.5°C) when treated with ABA (Chen & Gusta, 1983). This frost tolerance equals that of the most frost hardy winter rye lines, which shows high winter survival on the Prairies.

1.2. CBF regulon has an important role during cold acclimation

The variation in cold-tolerance among plant species relates largely to the rate and duration of the cold-acclimation process during fall (Limin & Fowler, 2006). This phase takes four to seven weeks in winter rye and wheat as they start to acclimate at higher temperature than the less cold-hardy winter barley. Barley only acclimates for three weeks leading to low accumulated freezing tolerance. During cold acclimation, the shoot apical meristem (SAM) transitions from vegetative to reproductive competence (Fowler *et al.,* 1996). In addition, a multitude of physio-biochemical changes occur in the plant (Limin & Fowler, 2006; Limin *et al.,* 2006), wherein expression of many cold-regulated (*Cor*) genes is activated (Thomashow, 1999). Among the factors inducing COR gene expression is a group of DNA binding transcriptional activators called C-repeat binding factors (CBFs). These factors bind to CCGAC motifs called C-repeat element (CRT) present in the *Cor* promoters, and thereby activate *Cor* expression which leads to increased freezing tolerance in the plants (Yamaguchi-Shinozaki & Shinozaki, 1994).

CBF genes have been identified in a wide range of plant species including cold tolerant plants, such as winter rye and winter wheat (Båga *et al*., 2007; Campoli *et al*., 2009; Choi *et al*., 2002; Mohseni *et al*., 2012; Schlegel, 2013), moderately cold tolerant plants, such as Arabidopsis (Jaglo *et al*., 2001; Medina *et al*., 1999) and cold susceptible plants, such as rice and tomato (Cao *et al*., 2008; Dubouzet *et al*., 2003; Ito *et al*., 2006; Zhang *et al*., 2004). The importance of CBFs in cold-tolerance was initially shown by transgenic studies in Arabidopsis (Medina *et al*., 1999) and later confirmed by similar studies in wheat and barley (Soltész *et al*., 2013; Takumi *et al*., 2008). To date, more than sixty CBF genes have been reported for hexaploid wheat (Mohseni *et al.,* 2012). Since wheat is an allohexaploid plant made up of three genomes (A, B and D), the wheat CBF genes represent about 20 different homeologs. The functional properties of CBFs and sequences flanking the CCGAC motif determine the strength of the protein/DNA interaction (Jain, 2013). Theoretically, a plant will be more cold tolerant if CBF genes are highly expressed and can efficiently bind to the CRT motifs of COR promoters (Kume *et al.,* 2005).

Even though CBF regulons have been identified in a wide array of plants, the number of genes involved in this regulon varies (Lata & Prasad, 2011; Nakashima & Yamaguchi-Shinozaki, 2005; Zhang *et al*., 2004). Irrespective of whether a species belongs to a cold tolerant or cold susceptible group of plants, the CBF regulon may include a few to hundreds of genes (Carvallo *et al*., 2011; Maruyama *et al*., 2004; Zhang *et al*., 2004). The CBF regulon in winter wheat is

assumed to be quite large based on transcriptome analysis studies (Gulick *et al.*, 2005; Laudencia-Chingcuanco *et al*., 2011). But the size of the CBF regulon is not the only determinant of the cold tolerance level in plants. A more important factor is the expression levels of CBF genes and their target genes during cold acclimation. For example, cold hardy winter wheat variety Mironovoskaya 808 shows a two and a half times higher expression of genes belonging to the CBF regulon than the cold sensitive variety Chinese Spring (Kume *et al*., 2005).

CBF genes located on the group five chromosomes in rye, barley, diploid einkorn wheat and hexaploid bread wheat are associated with quantitative trait loci (QTL) for cold-hardiness and winter survival (Båga *et al*., 2007; Campoli *et al.*, 2009; Miller *et al.*, 2006; Skinner *et al.*, 2005). The major frost-resistance region in hexaploid wheat is *Fr-A2* on chromosome 5A, which carries about 20 CBF genes. Most of the *Fr-A2* CBF genes have similar primary sequences (Badawi *et al*., 2007) and belong to Group III and Group IV subclades of the CBF phylogenetic tree (Mohseni *et al*., 2012; Jain, 2013). Generally, a higher level of CBF genes encoded by *Fr-A2* allele is associated with higher freezing tolerance among winter wheat accessions (Sutton *et al.*, 2009). Differences in CBF functional properties are also expected to contribute to differences in cold tolerance. One such example is *CBF12* in diploid *T. monococcum,* which encodes a functional protein in the cold-hardy *cv.* G3116, whereas a non-functional CBF12 is produced in the less cold hardy relative DV92 (Knox *et al*., 2008). The inactivation of CBF12 in DV92 is due to a mutation causing loss of five amino acids within the DNA binding AP2 domain (Knox *et al*., 2008). Similarly, a single amino acid difference within the AP2 domain encoded by one *CBF12* allele of wheat *cv.* Cappelle Desprez leads to a drastic reduction in DNA binding capacity as compared to the functional Norstar *CBF* allele (Jain, 2013). Several studies during the last 15 years confirm that CBFs play an important role in cold acclimation (Cook *et al.*, 2004; Gilmour *et al*., 1998; Lee & Thomashow, 2012; Novillo *et al*., 2007). However, only a few studies have analyzed the functional properties of CBFs produced in cereals (Ito *et al*., 2006; Jain, 2013), and this area of research needs to be investigated further to fully understand which CBF properties are most critical for freezing tolerance.

1.3. Hypothesis

Structural differences exist in CBF proteins encoded by group five chromosomes in winter wheat.

1.4. Objectives

- Optimize cloning, expression and purification of the CBF proteins encoded by the group 5 chromosomes homeologous regions of winter wheat.
- Assess the suitability of recombinant CBF proteins for functional analyses.

CHAPTER 2

LITERATURE REVIEW

2.1. Evolution of Poaceae

Wheat is a member of Pooideae subfamily of the grass family Poaceae, which also includes other economically important crops, such as rice and corn. Together, these three crops provide more than 50% of the calories consumed by humans (Raven & Johnson, 1995). Members of Poaceae cover almost 20% of the world land mass and are distributed from the hot tropics to the cool temperate regions (Clayton, 1981; Shantz, 1954). The origin of the taxon occurred almost 60-55 mya during the Paleocene to early Eocene eras, based on the age of the oldest fossil records of grass pollen (Jacobs *et al*., 1999). The Poaceae species became the dominant flora in the northern hemisphere by the late Tertiary era (Strömberg, 2002) and diversification occurred based on the climatic conditions of the earth (Kuper *et al*., 2008). With the advancement of molecular biology tools, scientists have concentrated their studies on the organelle and nuclear genes to decipher the evolution of grasses. Sequence homology among chloroplast genes such as, *Rps4* (Nadot *et al*., 1994; Nadot *et al*., 1995), *RbcL* (Barker *et al.*, 1995), *MatK* (Hilu *et al.*, 1999; Hilu & Alice, 2001), *NdhF* (Clark *et al.*, 1995), and nuclear genes including *GBSSI* (Mason-Gamer *et al*., 1998), *Acc1* (Fan *et al*., 2013), *Pgk1* (Fan *et al*., 2013) and Phytochrome B (Mathews *et al*., 2000) have revealed the origin, evolution and the divergence of the 12 subfamilies of the Poaceae (GPWG II, 2012). Though the origin of the Poaceae coincided with the warmest climate in Earth's history (Early Eocene), the distribution and evolution coincided with one of the coolest climates that the earth has ever witnessed (Katz *et al.,* 2008), a shift known as, "Greenhouse to Icehouse Transition" (Zachos *et al*., 2001). This, resulted in an extreme requirement to adapt (Barker *et al*., 2001). During this time it became necessary for wheat to withstand colder climates and three cold related gene families, namely the ice recrystallization inhibitor or IRI protein family, fructosyl transferase or FT (not to be confused with *flowering locus T*) protein family and C-repeat binding factor or CBF evolved during this cooling period (Sandve *et al*., 2008; Sandve & Fjellheim, 2010; Sandve *et al*., 2011). Both CBF and FT have evolved in a Pooideae-specific manner (Hisano *et al.,* 2008). CBFs are the most important among these three groups of proteins for being transcription factors and thus regulating the expression of other cold responsive genes (Agarwal *et al.,* 2006). CBF genes are

also known for their roles against other abiotic stresses such as drought (Xue *et al.*, 2008; Xu *et al*., 2009). The CBF gene family may have evolved as an overall defense mechanism against abiotic stresses particularly low temperature (Sandve & Fjellheim, 2010). It has been postulated that divergence in the CBF gene family of the Pooideae occurred during the onset of Eocene-Oligocene colder climate as a survival strategy (Sandve & Fjellheim, 2010). This phenomenon can be observed in today's warmer world where cold tolerant crops such as winter wheat and winter rye have higher copy numbers of active CBFs, whereas, cold susceptible crops (rice, spring wheat and barley) either lack functional CBFs or have lower copy numbers.

The evolution of wheat parallels human evolution. About three million years ago (mya) three diploid wheat species bearing the A, B and D genomes diverged from a common ancestor almost at the same time when humans evolved from apes (Gill *et al.*, 2004). When the modern human originated about 0.2 mya, tetraploid wheat species emerged through naturally occurring crosses between *A. speltoides* and *T. urartu* (Golovnina *et al*., 2007; Fig. 2.1). Almost 15,000 years ago, mankind sowed the seed of civilization by domesticating hexaploid wheat in the warm Middle Eastern Fertile Crescent of modern day Iraq, Iran, Turkey and Syria (Harlan, 1992). The allohexaploid (2n=6x=42; AABBDD) bread wheat evolved in the Fertile Crescent from naturally occurring crosses among *T. urartu* Thum. Ex Gandil. (Source of A genome), *Aegilops speltoides* (Tausch) Gren. (Possible source of B genome) and *A. tauschii* Coss. (synonym: *A. squarrosa* Auct.; source of D genome) (Feldman, 2000; Gororo, 2001; Shewry, 2009; Talbert *et al*., 1998). Both spring and winter types of bread wheat have a monophyletic Mediterranean origin as inferred from analysis of nuclear rDNA sequences (Hsiao *et al*., 1995) and SSR markers (Dvorak *et al.*, 2012). Among the various methods through which cold tolerance in wheat was improved, the most effective was polyploidization and genome duplication (Fawcett *et al*., 2009; Lawton-Rauh, 2003; Soltis *et al*., 2008; Fig. 2.1) through genetic recombination (Akhunov *et al.*, 2003) which shaped the evolution of modern bread wheat (Feldman & Levy, 2005).

2.2. Wheat production on the Canadian prairies

One of the most significant cereals in modern agriculture is wheat, for which Canada ranks eighth in terms of production (37.5 million tonnes in 2013; www.statcan.gc.ca). Cultivated wheat is distinguished on the basis of genome complexity and includes hexaploid bread wheat

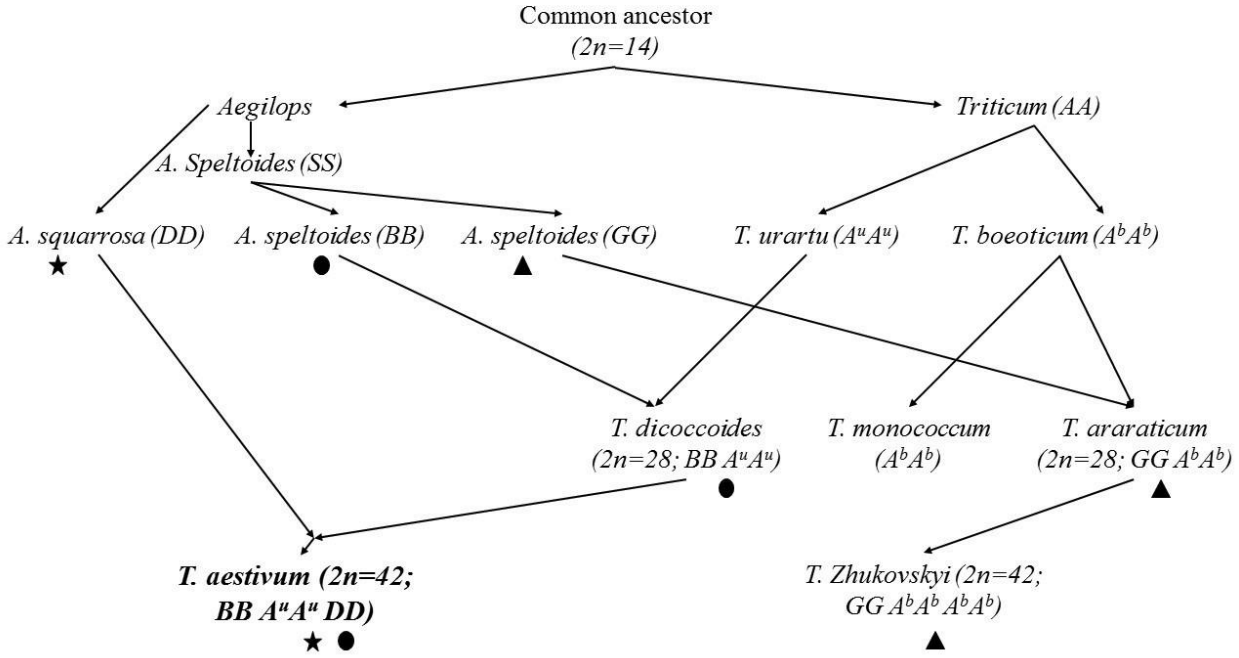

Fig. 2.1. Schematic representation of the origin and evolution of hexaploid wheat species. Letters within parenthesis signify each species representing their genome constituents. Oval, star and triangle shapes represent the path of genome inheritance. Adapted and modified from Golovnina *et al*., 2007.

(*Triticum aestivum* L.) and spelt wheat (*T. spelta* L.), tetraploid durum (*T. durum* L.) and diploid einkorn (*T. monococcum* L.) wheat. Durum and bread wheat dominate wheat production in Canada.

Wheat is a well-adapted crop that can be grown in a wide range of climates because of its spring and winter growth habit. Bread wheat is represented by both spring and winter types, but winter wheat production is relatively small on the Prairies (about 6 %) because of low winter-hardiness (LT_{50} >-24°C; Gullord *et al*., 1975; Gusta & Wisniewski, 2012). Of the 15 milling classes of wheat produced in Canada, only four are winter wheat: Canada eastern hard red winter (CEHRW), Canada eastern soft red winter (CESRW), Canada eastern white winter (CEWW) and Canada western red winter (CWRW). Some of the winter wheat varieties and a few low protein spring varieties are included in Canada western general purpose (CWGP) which are used for feeding purpose. Most of the winter wheat produced on the Prairies is grown close to the US border although the crop is at risk for winter-kill in these areas. In the 1984/85 season, nearly all winter wheat on the Prairies was killed by frost (http://www.princeton.edu/~ota/disk1/1989/8917/891706.PDF). In 2012, severe flooding in the spring in addition to winter-kill reduced the winter wheat production acreage by 46% compared to the previous year (http://www.agriculture.gov.sk.ca).

Winter wheat produced on the Canadian Prairies is seeded during the fall season into standing canola stubble. During the winter, most of the plant tissues are killed by frost, except the crown tissue that survives below the soil level, if winter conditions are mild. During spring, new leaves and roots emerge from the crown and plant growth continues with maturity in late summer. Winter wheat is a good weed competitor, may escape *Fusarium* head blight and can produce approximately 30% higher yield than spring wheat if the crop successfully overwinters. Unfortunately, winter kill often leads to reduction in productivity and yield. Limited genetic variation for freezing tolerance in genotypes used for breeding has led to a plateau in the development of hardier winter wheat cultivars (Fowler, 2012). Hence, more in depth knowledge about the underlying mechanisms of cold hardiness in winter wheat will be useful to develop new strategies to incorporate enhanced LT tolerance in future varieties.

2.3. Winter hardiness in cereals

The two most important factors affecting plant growth and crop production globally are temperature and water availability. Sub-tropical crops such as rice, are chilling sensitive and are easily damaged by cool (6-12°C) temperatures (Jaglo *et al.*, 2001) and therefore only cultivated where daily temperature exceeds 18°C (Charles & Harris, 1972). In temperate climates, the effects of cool or low temperature induced by seasonal or geographical distribution largely determines plant survival and productivity. Temperate cereal crops grown in Canada (e.g. rye, wheat and barley) have, in contrast to rice, evolved to withstand cool temperatures at all stages of plant growth. However, defense against frost is not an intrinsic property, but must be acquired during cold acclimation (Larcher, 2003; Thomashow, 1999). This process occurs in the fall when newly established winter cereals become exposed to low, but non-freezing temperatures and lasts for weeks before maximum frost-tolerance is attained. Cold acclimated plants can survive freezing down to specific temperatures, as well as, fluctuating temperatures within that limit. These plants are thus referred to as winter-hardy (Humphreys, 1989). As the ancestors of Canadian temperate crop plants originated from southern latitudes, the winter cereal cultivars possess a relatively low level of winter-hardiness as compared to native species in Canada.

Winter rye (*Secale cereal* Linn.) is the most frost-tolerant cereal (*cv.* Puma, LT_{50} -33°C) for the temperate climates and can be cultivated in cold climates and on poor soil types where production of other cereals is unfavorable (www.uvm.edu). At the other extreme, the diploid (2n=2x=14) barley (*Hordeum vulgare* Linn.), which originated from *H. spontaneum* Koch and was domesticated independently in the Fertile Crescent (Badr *et al*., 2000) and in the Himalayan region (Dai *et al*., 2012) has poor winter survival. Winter barley is the least frost-hardy winter cereal grown in Canada, (LT_{50} > -15°C; Kosová *et al*., 2008) and is not suited for production on the prairies. Winter survival potential of winter wheat lies in between winter types of rye and barley, with LT_{50} value of -23°C for the most cold-hardy variety Norstar (Ganeshan *et al*., 2009; Fowler *et al*., 1995).

Besides winter frosts, freezing temperatures can also occur during the vegetative growth period and lead to substantial crop damage or plant death depending on timing, intensity and duration of frost (Bélanger *et al*, 2005; Brown & Blackburn, 1987; Chinnusamy *et al*., 2007). Spring seeded cereal crops are particularly vulnerable to frost-damage early in the spring and at the flowering and grain filling stages. A severe reduction in grain set and yield is often the

outcome when frost nights occur around anthesis as both the male and female reproductive organs of cereals are very frost sensitive (Thakur *et al.* 2010). For overwintering cereals with moderate frost-hardiness such as winter wheat, frost spells occurring in the early spring is also a threat to survival of winter cereals in Canada. In the early spring, the crown tissue has generally exhausted all of its accumulated cold tolerance in the fall and has little resistance to freezing temperatures that are even a few degrees below zero. In addition, wheat plants cannot cold acclimate in the spring due to the long day conditions (Trevaskis *et al.,* 2007). Thus, a low de-acclimation rate during winter is important for winter survival and to ensure adequate freezing tolerance remains in the early spring to protect against sudden frost. However, very little is known about factors regulating the de-acclimation rate in plants.

To utilize the full potential of cereal production in Canada, it is necessary to enhance frost tolerance in new winter wheat cultivars. One step towards this goal will be to better understand the key factors and genetics behind frost tolerance in cereals and utilize this knowledge to enhance field survival of winter wheat. These studies may also be beneficial to find methods to reduce the effects of low temperature stress occurring during the early stages of plant establishment in the spring, as well as during anthesis later in the season. In this review, the known factors associated with cold tolerance in plants will be summarized. Special emphasis will be placed on the available cold-hardiness information for wheat with reference to rye which better cold tolerance and barley which is cold susceptible.

2.4. Physiology of cold tolerance in plants

The plasma membrane is considered to be the perception site of the cold signal (Uemura *et al*., 2006), as well as, the primary injury site (Steponkus, 1984). During cold acclimation, changes to the membrane lipid composition occur that lead to increased cryo-stability (Steponkus & Webb, 1992). A high level of desaturation of membrane lipids increases the membrane fluidity (Ouellet & Charron, 2013; Yoshida, 1984; Yoshida & Uemura, 1984). However, recent studies have shown that membrane rigidification (inflexibility) is important for cold survival, as it leads to changes in homeostasis through the signal transduction pathways (Örver *et al.*, 2000; Fig. 2.2, 2.3). Low temperature induces proteomic changes which lead to adaptive changes in plasma membrane during cold acclimation. In wheat, cold acclimation results in changes in the constituents of the plasma membrane (Bohn *et al*., 2007). Plasma membrane associated proteins

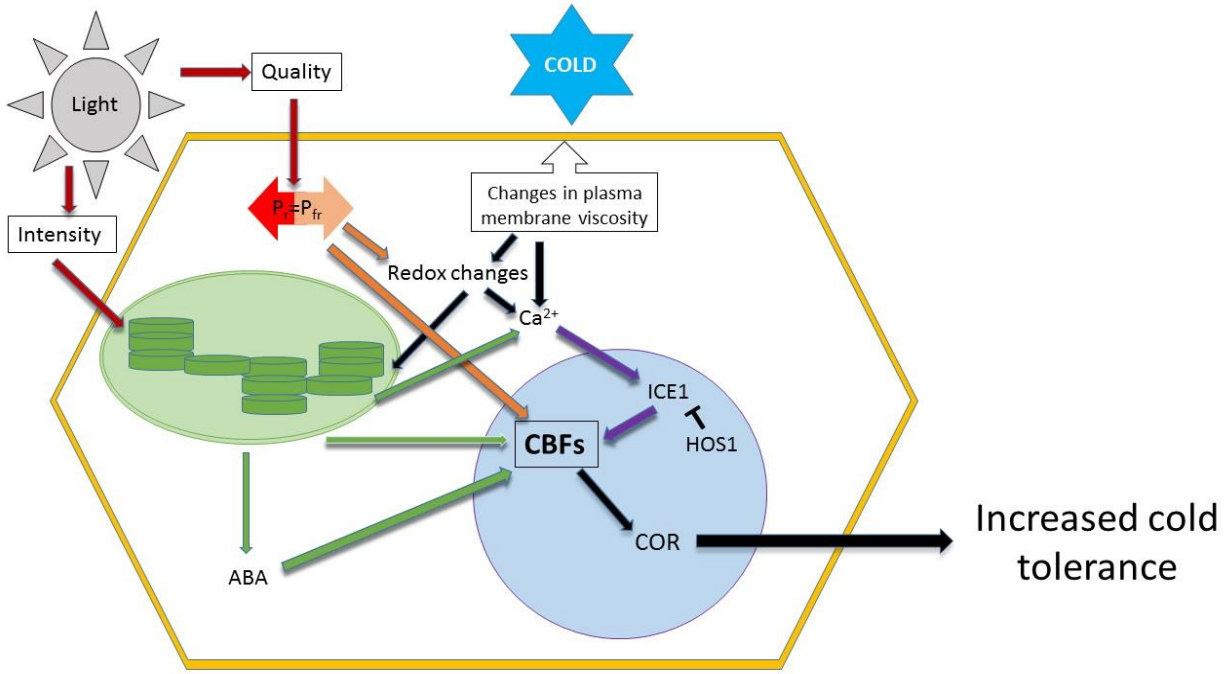

Fig. 2.2. Diagrammatic representation of the changes in plant homeostasis associated with cold acclimation. A controlled and coordinated signaling and gene expression mechanism involving light, plant hormones, Ca^{2+} and redox signals are required for cold acclimation (Monroy *et al*., 1993; Solanke & Sharma, 2008; Theocharis *et al*., 2012). The plasma membrane is the primary site of cold injury and has the ability to undergo compositional changes in cold-hardy species leading to increased cryo-stability (Steponkus, 1984). Changes in light intensity and quality during the winter leads to changes in photosynthetic efficiency and redox potential (Neff *et al*., 2000). An elevated cytosolic Ca^{2+} level occurs upon cold exposure and induces signaling cascades affecting expression of nuclear genes including CBF and COR genes. An increased cold tolerance is the result of increased COR expression. Green arrows indicate changes caused by photosynthetic apparatus; orange arrows indicate changes induced by phytochrome mediated pathway (Li *et al*., 2011); blue arrows indicate pathways triggered by changes in Ca^{2+} influx; black arrows represent effects of CBF induction. P_r - Phytochrome red; P_{fr} - Phytochrome far red; ICE - Inducer of CBF expression; HOS - High expression of osmotically responsive gene; CBF - C-repeat binding factors; COR - Cold regulated genes.

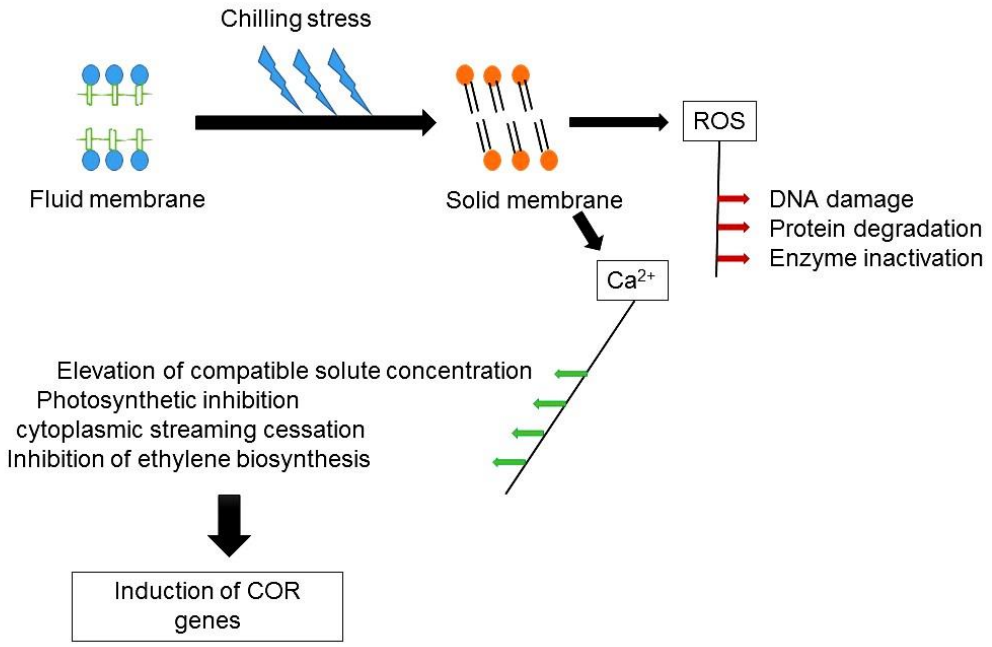

Fig. 2.3. Physical and cellular responses during cold stress. Response to chilling stress leads to membrane rigidity resulting in ROS and Ca^{2+} signaling. The change in homeostasis ultimately leads to induction of COR genes and increased cold tolerance.

belonging to membrane transport, vesicle trafficking, cytoskeleton and microdomain associated proteins were reported to be changed during cold acclimation (Minami *et al*., 2009). In Arabidopsis, plasma membrane associated phospholipase-δ increases during cold acclimation period (Li *et al.*, 2004). A plasma membrane associated temperature induced lipocalin-like protein family (TIL) has a positive influence on frost tolerance (Kawamura & Uemura, 2003; Takahashi *et al*., 2013) and over-expression of this protein leads to enhanced survival upon exposure to freezing temperature (Uemura *et al.*, 2006). Similar changes were also observed in the lipid constituents of plasma membrane. A 13-16% increase in free sterols has been observed along with a decrease in glycolipid constituents, especially in the amount of sterol glucoside and acetylated sterol glucoside in the plasma membrane of cold acclimated wheat plants (Bohn *et al*., 2007). An increase in the concentration of compatible solutes such as glycine betaine and oligosaccharides has also been observed in plants exposed to cold stress (Maruyama *et al*., 2009). In Arabidopsis, CBF3 is involved in the production of galactinol and raffinose biosynthesis, which act as cryo-protectants (Taji *et al*., 2002). In wheat, glycine betaine is over-produced to protect the plasma membrane under cold stress (Zhang *et al*., 2010).

2.5. Cold stress and cold signaling in plants

Cold induced injuries to the plasma membrane are often caused by severe dehydration and/or ice crystal formation within cells (Ingram & Bartels, 1996; Pearce, 2001). To reduce membrane vulnerability to frost damage, cold-tolerant plants have the ability to alter the lipid composition of the membrane to provide increased cryo-stability (Steponkus, 1984; Fig. 2.2; 2.3). During the initial cold stress, plants increase their cytosolic Ca^{2+} and reactive oxygen species (ROS; e.g. H_2O_2) levels (Chinnusamy *et al.*, 2007; Fig. 2.2; 2.3), which in turn activate a cascade of signaling pathways that ultimately affects expression of cold-regulated (COR) genes in the nucleus (reviewed by Heidarvand & Amiri, 2010; Fig. 2.2). Cold-induced secondary messengers such as Ca^{2+} or ROS might also be involved in inducing changes to the phytohormone levels in plants under cold stress (DeFalco *et al*., 2010; Fig. 2.2). ABA is one phytohormone that increases cold hardiness in winter rye and wheat cell suspension cultures (Chen and Gusta, 1983). The role of ABA in conferring cold tolerance was later confirmed by studies of the ABA-insensitive mutant *abi-1* of Arabidopsis (Mäntylä *et al*., 1995). The action of ABA is likely to be mediated through ABA-induced proteins binding to *cis*-regulatory ABA-

responsive elements (ABRE) carrying the conserved G-box motifs (CACGTGGC) present in the promoters of many COR genes (Choi *et al*., 2000). Other phytohormones such as auxins, gibberellins, cytokinins and ethylene might also have some effect on cold-tolerance (Jeon *et al*., 2010; Kosová *et al*., 2012; Shibasaki *et al*, 2009; Yu *et al.*, 2001), but they have not been studied in cereals as extensively as ABA.

Early studies on cold acclimation postulated that several nucleoproteins and structural proteins might play some role in enhancing cold tolerance (Weiser, 1970). Recent transcriptome, proteome and metabolome analysis of plants have confirmed that plants undergo a large set of transcriptional, biochemical and morphological changes during cold acclimation (Hughes & Dunn, 1996; Knight & Knight, 2012; Vágújfalvi & Tischner, 1999; Gilmour *et al*., 1998; Guy & Haskell, 1987). Epigenetic changes are also found to effect cold hardening and frost tolerance in plants (Kocsy *et al*., 2010; Chinnusamy *et al*., 2007; Uemura & Hausman, 2013).

Quantitative gene expression analyses show several COR genes are expressed differentially and tissue specifically during cold acclimation in wheat (Ganeshan *et al*., 2009). Among the cold-inducible proteins are antioxidants (Baek & Skinner, 2003), antifreeze proteins (Griffith *et al*., 1992) and enzymes involved in respiration and metabolism of carbohydrates (Partelli *et al*., 2010) and lipids (Wei *et al*., 2006),. The main function of these proteins is to help plants cope with the chilling and freezing stresses by protecting enzymes and membranes (Gong *et al*., 2002; Thomashow, 1999). For example, anti-freeze proteins provide tolerance to cellular dehydration caused by apoplastic freezing (Thomashow, 1999; Thomashow, 2010).

2.6. Vernalization requirement and its contribution to cold tolerance

Winter cereals need a long exposure to cold but non-freezing temperature to become flowering competent, which is referred to as vernalization (Chourd, 1960), which acts as an epigenetic memory in plants (Amasino, 2004). Spring varieties, on the other hand, flower without prior exposure to cold. The requirement for vernalization in overwintering plants is a necessary adaptation to produce seeds under favorable conditions (the following summer) and it is also needed to acquire maximum frost hardiness during cold acclimation (Prásil *et al*., 2004).

Vernalization in wheat is mediated by VRN genes, which regulate low temperature tolerance through their effect on the phenological developmental rate at the shoot apical

meristem (Mahfoozi *et al*., 2000). There are three VRN genes that provide winter hardiness in wheat: *Vrn1, Vrn2* and *Vrn3* and interactions can be observed among these genes (Galiba *et al.,* 2009). A fourth vernalization gene, *Vrn4 (VrnD4),* on chromosome 5D (Kato *et al*., 2003) is only responsive during exposure to a short vernalization period (Yoshida *et al*., 2010). The *Vrn1* encodes a MADS box transcription factor (Trevaskis *et al.,* 2003) and is the main determinant for vernalization requirement as confirmed by a *Vrn1* deletion mutant of *T. monococcum* that remains in vegetative phase for an indefinite period of time (Shitsukawa *et al*., 2007). Among the three *Vrn1* alleles in hexaploid wheat, *Vrn-A1* has a dominant role in reducing the vernalization requirement and initiating flowering (Loukoianov *et al*., 2005). The dominant spring type wheat carries one or several *Vrn1* alleles and evolved from the ancestral winter type by acquisition of mutations within the *vrn1* first intron (Fu *et al*., 2005) or in the promoter region (Pidal *et al*., 2009; Yan *et al.,* 2003) rendering constitutive expression of the *Vrn1*.

It has been proposed that VRN1 interacts with VRN2 and VRN3 to provide frost tolerance in wheat by activating the expression of CBFs (Galiba *et al.,* 2009; Fig. 2.4). VRN3 is a homologue of Arabidopsis flowering locus T (FT; Yan *et al*., 2006) and is up-regulated by long days thus activating expression of the *Vrn1 (*Li & Dubcovsky, 2008). The *Vrn2* on the other hand is down-regulated during short days and functions as a flowering repressor (Trevaskis *et al*., 2007; Yan *et al.,* 2004). Epistatic effects between *Vrn-A^m-1* and *Vrn-A^m-2* genes are also observed in *T. monococcum*, where the *Vrn-A^m-1* allele for spring growth habit is dominant over *vrn-A^m-1* allele for winter growth habit and *Vrn-A^m-2* allele for winter growth habit is dominant to v*rn-A^m-2* allele for spring growth habit (Tranquilli & Dubcovsky, 2000). In barley, vernalization can be explained by the two-loci *Vrn-H1/Vrn-H2* model based on the presence or absence of *Vrn-H2* and a winter vs. spring *Vrn-H1* (*HvBM5A*) allele (Zitzewitz *et al.,* 2005).

The genetically complex cold tolerance trait makes it difficult for breeders to select and distinguish between the cold regulated and developmental genes affecting this trait (Galiba *et al*., 2009). As winter wheat has a longer vegetative phase, it can maintain cold acclimation at a higher level for a longer period of time before saturation is reached (Prasil *et al*., 2004). Thus, developmental genes responsible for delaying transition from the vegetative to the reproductive phase play an important role in conferring cold tolerance in wheat and *Vrn1, Vrn2 and Vrn3* are considered to be the most important genes in this context. Other factors delaying floral transition

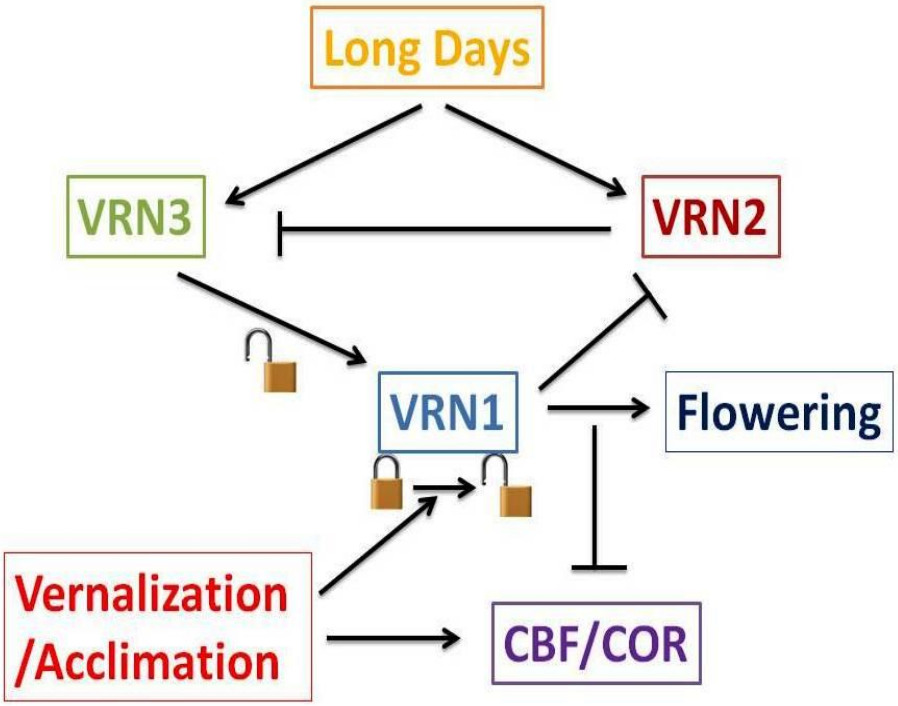

Fig. 2.4. Hypothetical pathway of interaction among VRN genes. *Vrn3* and *Vrn2* are up-regulated during long days. *Vrn2* inhibits *Vrn3* function. *Vrn3* up-regulates *Vrn1* and *Vrn1* in turn inhibits the expression of *Vrn2* and also the *Cbf/Cor* pathway. *Vrn3* and *Vrn1* function during vernalization, whereas, *Vrn2* is blocked during this process. Considering *Vrn1* as a lock, exposure to low temperature and activated *Vrn3* acts as a key to unlock its functionality (Adapted and modified from Galiba *et al.*, 2009).

in both vernalized and non-vernalized wheat plants are encoded from regions on chromosomes 1B, 2A, 2B, 6A and 7A as revealed by genetic mapping (Båga *et al*., 2009). An additional locus on chromosome 4A delays floral transition only in vernalized plants (Båga *et al*., 2009).

2.7. Natural variation in cold tolerance

QTL analyses of traits in plants have led to the identification of genes causing trait variation and extended our understanding of the dynamics and importance of different naturally occurring alleles for a particular trait (Alonso-Blanco *et al*., 2009). Recent forward and reverse genetic studies of the model plants like Arabidopsis have shown that cold acclimation is a complex genetic trait involving a large number of genes and pathways (Hannah *et al*., 2005; Sanghera *et al*., 2011). In addition, studies on natural variation within populations have also lead to identification of QTLs, as well as, novel genes that play a role in cold acclimation (Toth *et al*., 2003). Interaction with biotic and abiotic factors might influence the generation of novel genes through random mutations and these mutated novel genes can transiently be maintained within a population (Salvaudon *et al*., 2008). Identifying these important candidate genes present in a specific QTL is a better approach because it is less time consuming (Edae *et al*., 2013). These kinds of studies could link the phenotypic variations with polymorphic traits such as cold tolerance and acclimation, thus helping in identification of candidate genes (Wang *et al*., 2008). It is well-known that wild relatives of wheat (Zaharieva *et al*., 2001), rice (Liu, 2003), chickpea (Singh *et al*., 1995), tomato (Bolger *et al.*, 2014) and other cultivated crop plants possess higher genetic variation than the cultivars of these species. Thus, identification of candidate genes through sequencing of the wild species is another way of identifying gene variants that could possibly be used to develop improved stress tolerant varieties (Henry, 2013). In addition, the advancement of whole genome sequencing technologies makes the dissection of natural gene variation easier at the molecular easier.

One of the major regulons with an active role during cold acclimation is the CBF regulon. Because of their ubiquitous nature, CBF genes can be found in almost all plant species where they often form gene clusters. In a study of 48 Arabidopsis accessions, developed to capture most of the natural genetic variation, the CBF genes were found to be cold inducible irrespective of the cold tolerance level of the genotype (McKhann *et al*., 2004). Variation in cold acclimation ability in Arabidopsis is associated with the geographic region (Beck *et al.*, 2008; He *et al*.,

2007; Zuther *et al*., 2012). A recent study on Arabidopsis accessions that differ in cold tolerance indicated that there are naturally occurring variations present in their CBF genes, as well as, their promoter sequences (Kang *at al.,* 2013). Major QTLs of CBF gene clusters have also been identified on the group five chromosomes of wheat (Båga *et al*., 2007; Snape *et al.,* 2001; Toth *et al*., 2003; Fig. 2.5). Hence, studying the naturally occurring variation in wheat CBFs might provide more insight into the variation in cold tolerance levels in different accessions.

2.8. Genetic control of cold hardiness in winter wheat

The ability of winter wheat to survive sub-zero temperatures has made it a good system to study the complex *in planta* molecular mechanisms that allows it to become cold tolerant. The development of molecular markers, linkage analysis and mapping of QTLs are advantageous for studies of complex traits in plants. Using these technologies, genomic regions associated with various traits have been mapped (Båga *et al*., 2007; Taleei *et al.,* 2010), and in a few cases, candidate genes causing variation in trait values have been identified in wheat (Faris *et al*., 1999). It is known that temperate cereals such as winter wheat become tolerant to frost during the fall through the month-long cold acclimation process during which a large set of COR genes are induced to enhance frost tolerance throughout the plant (Thomashow, 1999). Expression of several COR genes is modulated (Thomashow, 1999) by the CBF group of transcription activators, which bind to a C-rich consensus motif present in the promoter region of the COR genes (Yamaguchi-Shinozaki & Shinozaki *et al*., 1994).

2.8.1. Physical mapping of CBF gene clusters

The first frost tolerance QTL in wheat was identified on chromosome arm 5AL and denoted as frost resistance (*Fr*) locus 1 (Galiba *et al.,* 1995). Later it was revealed that a second locus for frost resistance is located ~30 cM upstream of *Vrn1* in diploid and hexaploid wheat and denoted *Fr-A2* (Vágújfalvi et al., 2000). These are the major loci controlling frost resistance and winter-hardiness in diploid and hexaploid wheat (Båga *et al*., 2007; Knox *et al*., 2010; Miller *et al.,* 2006; Motomura *et al*., 2013; Vágújfalvi *et al.,* 2003; Fig. 2.5). *Fr-A1* coincides with the vernalization requirement locus *Vrn-A1* (Sutka *et al*., 1999) responsible for developing spring and winter habit in wheat (Fowler *et al.,* 1996). It was initially unclear whether the locus is a different gene from *Vrn-A1* or a pleiotropic effect of *Vrn1* (Fowler *et al*., 1995; Limin & Fowler,

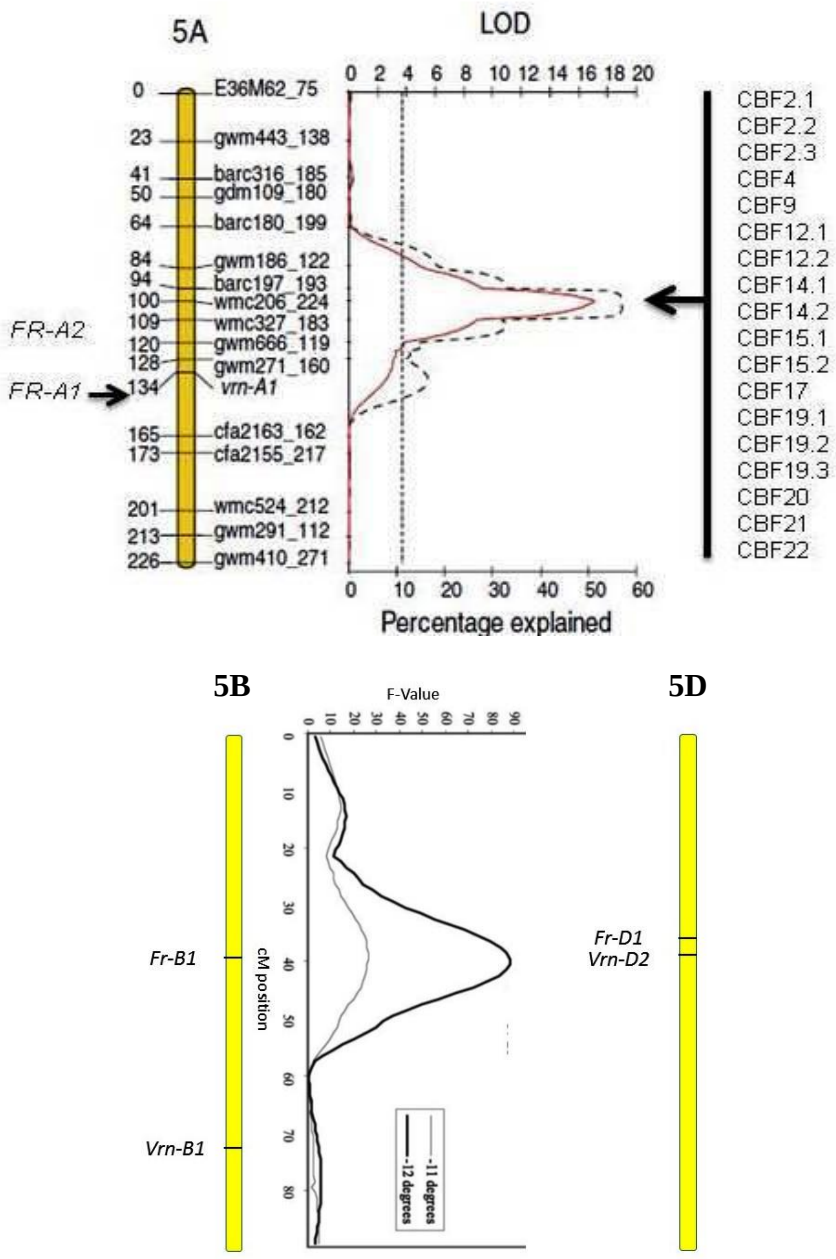

Fig. 2.5. Major frost tolerance QTLs in winter wheat on chromosome 5A (Båga *et al*., 2007), 5B (Toth *et al*., 2003) and 5D (Snape *et al*., 2001). A vast number of CBF genes underlie the *FR-A2* locus (Båga *et al*., 2007) and the *Fr-D2* locus (Båga, unpublished data). *Fr* and *Vrn* loci coincide on both chromosomes 5A and 5D, whereas, in 5B they are far from each other.

2006). Later studies have shown that allelic variation in *Vrn-A1* is adequate to determine the differences in freezing tolerance, and that *Fr-A1* QTL corresponds to *Vrn1* MADS box gene (Dhillon *et al*., 2010; Zhu *et al*., 2014).

Chinese Spring, a spring wheat line, showed increased cold hardiness when its chromosome 5A was replaced by that of winter wheat line Cheyenne carrying the recessive *VRN1* alleles (Eagles *et al*., 2011; Pugsley *et al.*, 1971). Because winter wheat has more CBF genes on the 5A chromosome, the reason for the freezing tolerance in Chinese Spring containing the 5A chromosome from Cheyenne could be because of increased transcription of those CBF genes (Vágújfalvi *et al.*, 2005). In Arabidopsis, increased freezing tolerance is a complex process and is only partially dependent on CBF regulon (Park *et al*., 2015). Compared to Arabidopsis, the wheat genome carries more CBF genes and cluster of CBF genes are located within the *FR-A2* QTL region (Båga *et al.*, 2007; Fig. 2.5). Some CBFs have also been identified on chromosomes 5B and 5D and in some genetic mapping populations these regions also appear as QTLs for frost tolerance (Toth *et al*., 2003). Therefore it may be suggested that even though CBFs from chromosome 5A are the key regulators in frost tolerance, CBFs from chromosome 5D also take part in frost tolerance tolerance to some extent. Similar to wheat, CBF genes have also been found at the *Fr-H1* and *Fr-H2* loci in barley (Stockinger *et al*., 2007).

2.9. Structure of CBF

CBF transcription factors, also known as DRE binding (DREB) factors, are characterized by the presence of a highly conserved DNA binding AP2 domain (Stockinger *et al*., 1997), consisting of a conserved 60-70 amino acid residues flanked at either sides by two signature motifs: the upstream signature motif CMIII-3 (PKK/RPAGRxKFxETRHP) and the downstream CMIII-1 (DSAWL/R) (Fig. 2.6; Allen *et al*., 1998; Jaglo *et al*., 2001, Zhu *et al*., 2013). In Arabidopsis, CMIII-3 is responsible for the determination of the DNA binding ability of the CBFs (Canella *et al*., 2010). Amino acid alignment of CBF sequences shows that wheat CBFs are similar to Arabidopsis, *Brassica napus*, rye and tomato CBFs at both the N-terminal and C-terminal ends with, irrespective of being produced in cold-tolerant or sensitive plants (Jaglo *et al*., 2001). To date, a total of 13 *Cbf* genes have been identified in the *T. monococcum* genome (Miller *et al*., 2006), and 65 CBF genes representing 27 paralogous genes with 1–3 homeologous copies each have been reported for hexaploid wheat (Mohseni *et al.*, 2012; Fig. 2.7).

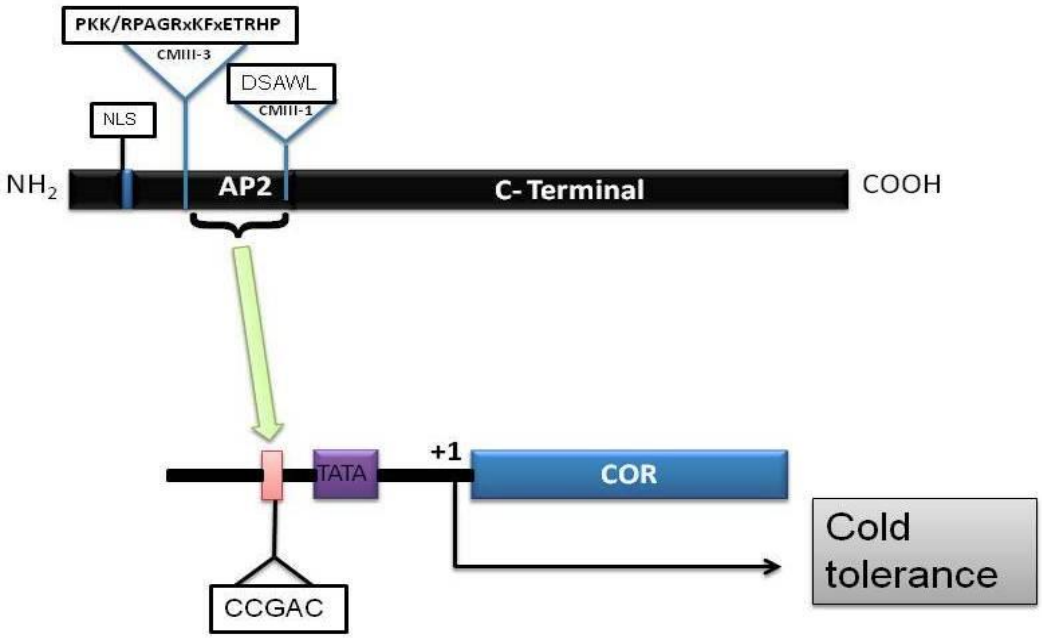

Fig. 2.6. A diagrammatic representation of CBF and its DNA target motif. Approximate location of nuclear location signal (NLS) and AP2 DNA binding domain flanked by signature sequences are shown (Based on Skinner *et al*., 2005).

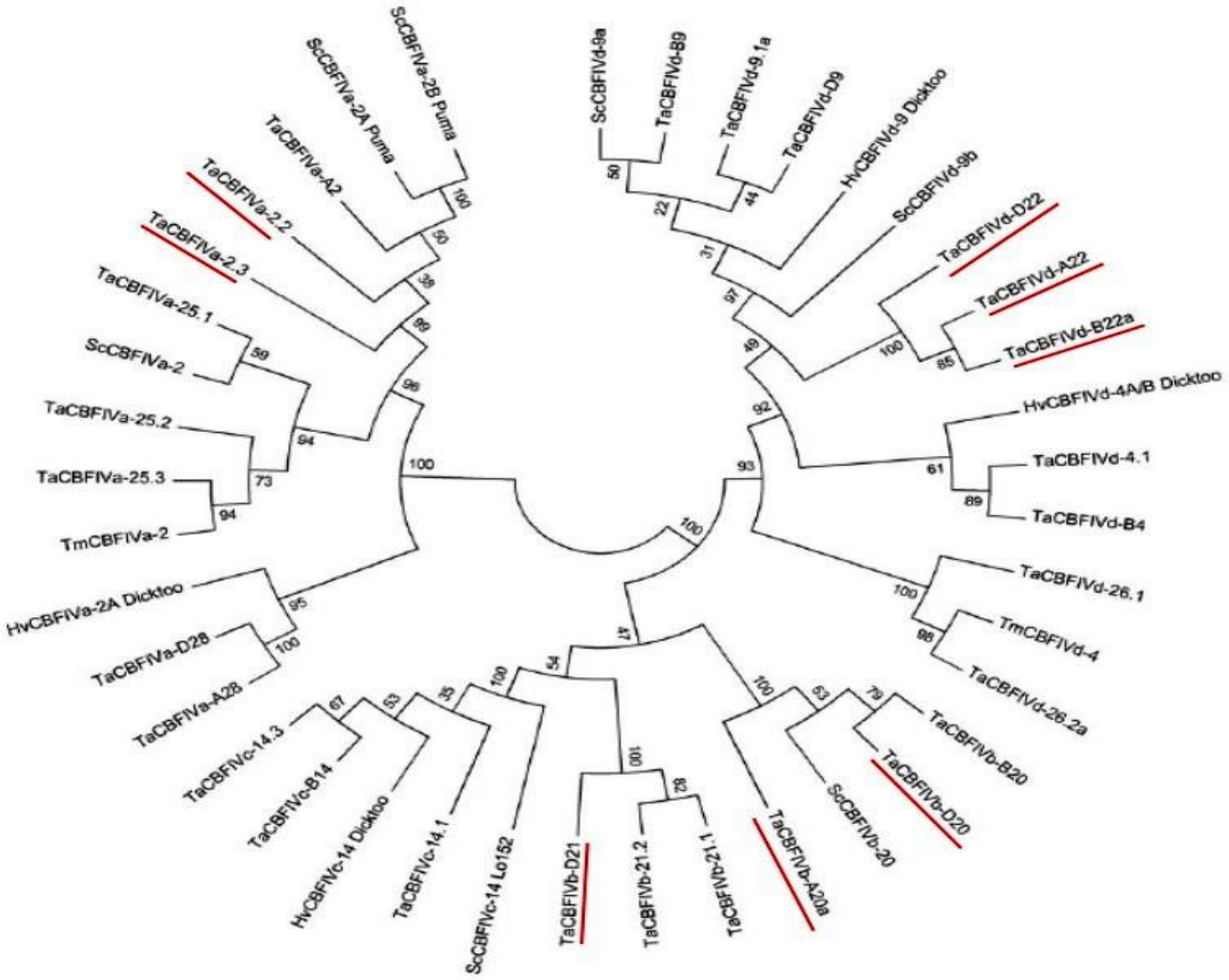

Fig. 2.7. Phylogenetic tree of sub-group IV wheat CBFs and their relationship with other Poaceae CBFs (Mohseni *et al*., 2012). Underlined CBFs were used in this study.

Badawi *et al.* (2007) suggested that six of 10 subgroups of the CBF gene family specifically belong to the Pooideae subfamily, that includes wheat. These sub groups are IIIc, IIId, IVa, IVb, IVc and IVd and they share a common monophyletic origin and that subgroups IIa and IIIb evolved before the divergence of the Poaceae subfamily (Badawi *et al.*, 2007).

2.10. Transgenic studies on CBFs

Upon initiation of cold temperatures, CBFs are expressed and bind to the T/ACCGACA/T motifs present in the COR gene promoters (Stockinger *et al*., 1997; Yamaguchi-Shinozaki & Shinozaki, 1994) and thereby induce COR gene expression. Several transgenic studies of different CBF genes confirm the genes enhance the cold tolerance level of plants at non-acclimating temperatures (reviewed by Lopato & Langridge, 2011). For example, over-expression of wheat *CBF2* in tobacco increased the cold tolerance level in the transgenic plants (Takumi *et al*., 2008). Over-expression of CBF3 and CBF related *Os*DREB2 in Arabidopsis had an enhanced level of cold tolerance (Gilmour, 2000; Kasuga *et al*., 1999; Liu *et al*., 1998). Transgenic rice over-expressing syngeneic *DREB1/CBF* also showed an improved tolerance to low temperature stress, but the plants also show a retarded growth phenotype (Ito *et al*., 2006).

Tobacco plants transformed with *GbCBF1* from cotton have lower electrolytic leakage upon freezing compared to wild type plants upon exposure to cold temperature, an indication of increased cold tolerance (Guo *et al*., 2011). Arabidopsis plants over-expressing a CBF obtained from arctic *Vaccinium myrtillus* and expressed under a constitutive 35S promoter showed better cold tolerance ability and constitutively activated *Cor* genes even in absence of cold (Oakenfull *et al.*, 2013). Overexpression of a CBF gene from cold tolerant blueberry cultivar Bluecorp in cold sensitive cultivar Legacy enhances the cold tolerance level in the transgenic plants (Walworth *et al.*, 2012). Transgenic barley lines over-expressing *Cbf14* and *Cbf15* obtained from hexaploid wheat showed enhanced cold tolerance (Soltész *et al*., 2013). These studies prove the importance of CBF transcription factors in enhancing cold/freezing tolerance levels in different species of plants. Over-expression of CBF genes in Arabidopsis resulted in a global change at the expression level of several COR genes (Jaglo-Ottosen *et al.*, 1998).

2.11. Factors influencing CBF expression and function

2.11.1. Regulation at molecular level

The DNA sequence of the CBF recognition site is simple however, the regulation of the DREB/CBF pathway is complex. The expression of CBF genes are induced by a group of transcription factors called, Inducer of Cold Expression or ICE (Chinnusamy *et al*., 2007; Zarka *et al*., 2003), which bind to the MYC recognition elements in the promoters of CBF genes (Fig. 2.8). Upon sudden cold exposure, a rapid 10-fold increase in *CBF* transcripts occur with the maximum level achieved after about two to 3 h exposure followed by a decrease in expression (Thomashow, 2010). A quick activation of ICE1 mediated through SIZ1-dependent sumoylation followed by inactivation due to HOS1-mediated ubiquitination and degradation. This may explain the cold treatment induced *Cbf* transcript accumulation (Thomashow, 2010; Fig. 2.8)., On the other hand, HOS15 is a histone modifier acting as a negative regulator of histone 4 acetyltransferase and plants with mutations in this gene show hypersensitivity to cold treatment (Zhu *et al*., 2008).

One of several factors involved in CBF signaling pathway is the calmodulin-like transcription activators (CAMTAs), which bind to promoters of certain CBF genes (Doherty *et al.*, 2009; Fig. 2.8). The CAMTAs might also be directly involved in downstream regulation of gene expression through the low temperature–induced cytosolic calcium signal transduction pathway (Eckardt, 2009; Fig. 2.8). The Sensitive to Freezing 6 (SFR6) protein is also implicated in CBF gene regulation (Knight *et al*., 2009), as shown from the reduced freezing tolerance level of *sfr* mutants in Arabidopsis (Warren *et al*., 1996). However, it appears that SFR requires additional factors for activity (Wathugala *et al.*, 2014). Cold acclimation specific genes (CAS) are potent targets of CBFs and help plants in cold acclimation. Recent studies have shown that even though similar CBFs are present in the cold susceptible species *Medicago truncatula* and cold tolerant species *M. falcata*, the difference between the cold tolerance and cold susceptibility is due to the difference in the copy number of CAS genes, with higher *Cas* copy number making *M. falcata* a cold tolerant species (Pennycooke *et al*., 2008).

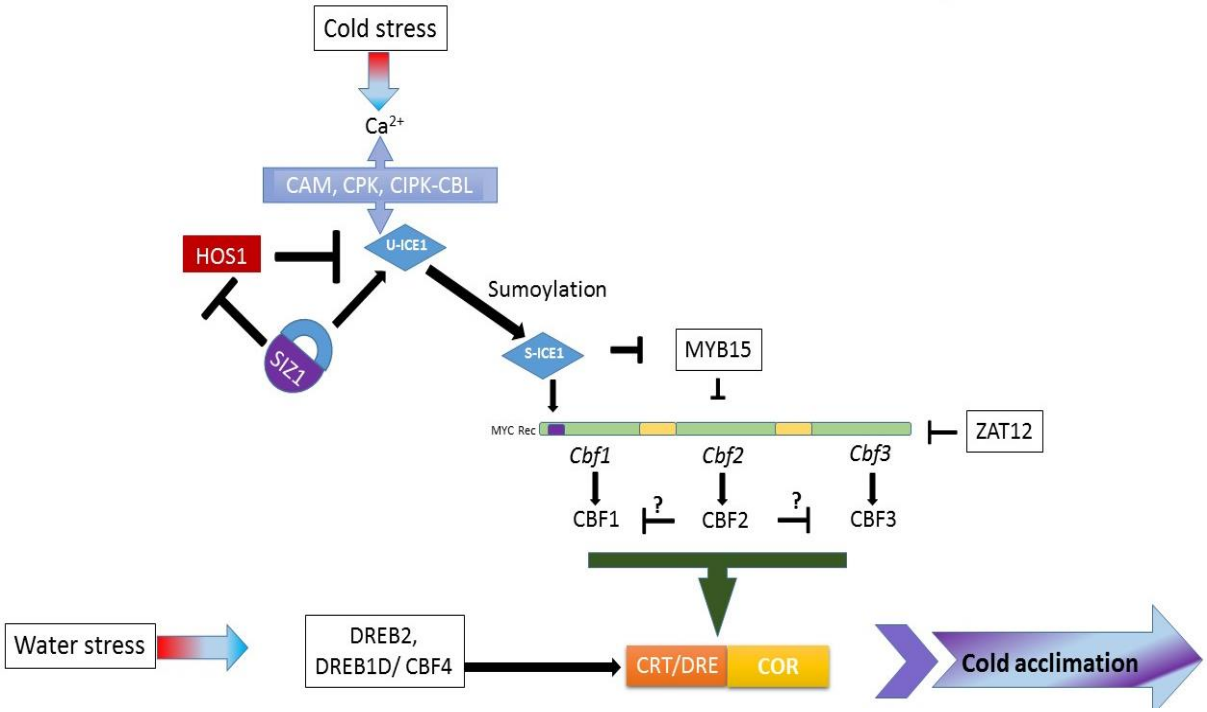

Fig. 2.8. A hypothetical model of CBF regulation in plants (reviewed by Miura & Furumoto, 2013). Cold stress leads to Ca^{2+} release into cytoplasm. SIZ1 sumoylates the unstable *Ice-1* gene product to stable and active form, which regulates the function of *Cbf* genes by binding to the MYC recognition factor present in the promoter region of the *Cbf* genes. MYB15 and HOS1 proteins negatively regulate the expression of CBFs. CBF2 has an antagonistic effect on the expression of CBF1 and CBF3. Upon expression, these CBF proteins act as transcription factors and regulate the transcription of *Cor* by binding to the C-repeat domain present at the promoter region of these genes. This eventually results in cold acclimation and enhanced cold tolerance. U-ICE1, Unstable ICE1; S-ICE1, Stable ICE1; CAM, Calmodulin; CPK, Ca^{2+}-dependent protein kinases; CIPK-CBL, Ca^{2+} sensor-associated protein kinases-calcineurin B such as Ca^{2+} sensors, HOS, High expression of osmotically responsive gene; ICE, inducer of CBF expression; DREB, dehydration responsive element binding factors; ZAT12, Zing finger protein 12; CRT; C-repeat; DRE; dehydration responsive element; CBF, C-repeat binding factors; COR, Cold regulated genes.

CBF promoter regions carry MYB recognition sites and binding of MYB transcription factors to these regions negatively influence the expression of *Cbf* (Agarwal *et al.*, 2006; Fig. 2.8). Upon exposure to cold, a differential expression pattern for *Cbf-3, Cbf-5, Cbf-6, Cbf-12, Cbf-14 and Cbf-19* was observed between frost resistant and sensitive wheat lines (Sutton *et al*., 2009). In a recent study in barley, expression of *Cbf9* and *Cbf14* was positively regulated by a pathway involving Phospholipase C and Phospholipase D, whereas, *Cbf12* expression was negatively regulated by these pathways (Marozsán-Tóth *et al*., 2015). During warm long days the PHYB photoreceptor interacts with transcription factors such as PIF4 and PIF7 (Leivar & Quail, 2011) and together suppress the expression of *Cbf* to conserve energy in Arabidopsis (Lee & Thomashow, 2012). PIF7 in Arabidopsis is also found to be a negative regulator of DREB1/CBF expression under the control of circadian clock (Kidokoro et al., 2009).

2.11.2. Regulation by physical factors

Light conditions, photoperiod, circadian rhythm and phytohormones also influence the expression pattern of CBFs (Egawa *et al.*, 2006; Kurepin *et al.*, 2013; Lee & Thomashow, 2012; Shi *et al.*, 2012b; Xia *et al*., 2009). Even though the genes required for vernalization and photoperiod are controlled through different pathways in winter cereals, they converge to influence the expression of LT induced genes (Trevaskis *et al*., 2007). After reaching the vernalization saturation point, plants become competent for reproduction. However, short day conditions in the fall prevents the developmental switch to flower production, thereby a higher level of expression of cold- responsive genes can be maintained (Fowler & Thomashow, 2002; Mahfoozi *et al*., 2000). Spring wheat contains the dominant alleles for photoperiod genes *Ppd-1* (Snape *et al.*, 2001) whereas winter wheat contains one or several copies of recessive alleles of *Ppd-1*, thus delaying flower development until the risk of freezing temperatures has passed in spring (Bentley *et al*., 2013; Kumar *et al*., 2012). Plants that grow in a low R:FR light ratio show increased expression of *COR15a*, *COR15b*, and *KIN1* genes, which are CBF targets in Arabidopsis (Franklin & Whitelam, 2007). CBF genes are positively regulated by light in Arabidopsis (Kim *et al*., 2002) and in the mangrove plant, *Aegiceras corniculatum* (Peng *et al.*, 2015). Surprisingly, the expression of CBF regulon in Eucalyptus is negatively regulated by light (El Kayal *et al*., 2006). This supports the observation that light quality, which varies between seasons, has a prominent role in the regulation of cold-inducible genes (Fig. 2.2; 2.3).

One of the major factors in cold acclimation is the interaction between the cold-responsive pathway and the circadian rhythm as observed from the extensive variation in cold regulated transcriptomes due to the disruption in the circadian clock in Arabidopsis (Bieniawaska *et al*., 2008). In Arabidopsis all three CBFs involved in cold acclimation- *AtCBF1*, 2 and 3 are regulated by the circadian rhythm (Fowler *et al*., 2005). An Arabidopsis triple mutant (*d975)* for all three alleles of the circadian clock gene *Pseudo Response Regulator* (*PRR*) had an elevated *Cbf* expression, along with a higher level of proline and raffinose accumulation, showing the antagonistic effect between these two pathways (Nakamichi *et al.*, 2009). The circadian rhythm regulating Evening Element (EE) and evening element like (EEL) genes are also found to be involved in the regulation of cold acclimation pathway and they function in combination with the ABA responsive element-like ABREL motifs (Mikkelsen & Thomashow, 2009). Studies on a double mutant plant revealed that in Arabidopsis the *Circadian Clock Associated* gene *CCA1* and *Late Elongated Hypocotyl* (*LHY*) genes together positively regulate the expression of the CBF pathway (Dong *et al*., 2011). Transgenic studies have shown that both the CBF regulon and the circadian rhythm have effects on each other. Over-expression of a peach *Cbf* in apple resulted in short day dependent dormancy even when grown at higher temperature (Wisniewski *et al*., 2011).

Apart from environmental factors, regulation of CBF expression also depends on the developmental stage of a plant (Al-Issawi *et al*., 2013). An increase in the expression level of *Cbf14* was observed upon exposure to low temperature during and after ear development of wheat (Al-Issawi *et al*., 2013). The CBF dependent cold acclimation pathway also affects the hormonal regulation in plants. In Arabidopsis, the cold inducible CBF1 increases the accumulation of a nuclear growth repressing protein class, known as DELLA repressor, which reduces gibberellic acid (GA) activity by stimulating the expression of GA inactivating *GA2-oxidase* and as a result, plants that over-express *Cbf1* show characteristic dwarfism (Achard *et al*., 2008).

2.12. Production of recombinant proteins in *E. coli*

Transcription factors regulating gene expression are generally produced in very low amounts (Harrison *et al*., 1991), which makes it nearly impossible to purify these proteins in mg amounts from plants tissues. However, isolating sufficient quantities of CBF proteins is not a

limitation if an expression system is used for protein production. Both prokaryotic and eukaryotic systems can be used for expression of recombinant proteins. However, eukaryotic systems are expensive and time consuming to use (Philipps *et al*., 2005), whereas *E. coli* based prokaryotic expression systems are relatively easy and inexpensive (reviewed by Baneyx, 1999; Carrier *et al*., 1983; Choi & Lee, 2004; Rosano & Ceccarelli, 2014). *E. coli* expression systems based on the *lac* operon and induction using IPTG are most commonly used for production of recombinant proteins. Unlike lactose, IPTG is not metabolized by bacterial cells and thus IPTG concentration is unchanged in media after addition and production of recombinant proteins remains unhindered (reviewed by Donovan *et al*., 1996). Many expression vectors and host cells with different properties are commercially available for protein production. However, there is no general expression system suitable for all types of recombinant proteins and optimization of expression conditions are generally needed.

Prokaryotic systems often provide sufficient amounts of properly folded and active recombinant protein for functional and structural studies. This is important for functional studies, which require proteins in their native form. However, a common problem is accumulation of over-produced protein within insoluble inclusion bodies, which require denaturing conditions to release the trapped proteins (Carrier *et al*., 1983; San-Miguel *et al.*, 2013; Valax & Georgiou, 1993). Several strategies to reduce the aggregation of recombinant proteins have been reported in the literature. These include, use of a cold inducible expression system (Qing *et al*., 2004; Schein & Noteborn, 1988), induction at low cell density, growth at low temperature (San-Miguel *et al*., 2013), using low concentration of isopropyl-β-D-1-thiogalactopyranoside (IPTG) for induction (Winograd *et al*., 1993) or co-expressing thioredoxin to assist solubilization of the recombinant protein (Lavallie *et al.,* 1993; Sahdev *et al.,* 2008; Sørensen & Mortensen, 2005a,b) may lower the aggregation problem. Growing *E. coli* cells at lower than 37 °C can effectively reduce inclusion body formation and protease activities (Fakruddin *et al.,* 2013). However, it may increase the chance of protein aggregation due to longer time required for cell culture at lower temperature or it may decrease the amount of protein produced (Fakruddin *et al*., 2013; Villaverde & Carrió, 2003). For further purification, an ion exchange and/or size exclusion chromatography step can be added to obtain protein preparations with higher purity. To remove excess salt from purified protein fractions, desalting is necessary and can be done using

commercially available desalting columns or by dialyzing the purified proteins against a buffer with low salt concentration.

To facilitate down-stream purification of over-produced protein from *E. coli* extracts, a tag is generally encoded as part of the desired protein. The most commonly used tags are S- and Histidine-tags (His). His-tags have a strong affinity towards metal ions, hence, metal affinity chromatography can be used to purify the recombinant protein of interest from a cell extract. In a previous study, wheat CBFs were expressed as *TrxHisS-CBF* fusion proteins composed of thioredoxin tag followed by His and S tags and the CBF protein (LaVallie *et al.*, 1993). The presence of the His-tag attached at the N-terminal allowed purification of the fusion protein from soluble *E. coli* extracts by Ni^{+} affinity chromatography. Dialysis was used to desalt the purified protein prior to downstream analyses. Although some of the recombinant CBFs from winter wheat *cv*. Norstar showed tendencies to aggregate in *E. coli* (Jain, 2013) many CBFs can be produced in native and active form (Knox *et al*., 2008; Skinner *et al.*, 2005).

2.13. Methods to study protein/DNA interactions

Studies of CBF functionality require a target DNA sequence to which the transcription factor will bind. An often used binding motif is one of the two CRT (CCGAC) motifs present in the *Wcs120* promoter (Ouellet *et al.* 1998). The *wcs120* gene family is one of the wheat COR genes which is induced by CBF expression (Houde *et al*., 1992a). The *Wcs120* gene family produces highly abundant heat-resistant proteins that vary from 10 to 200 kDa in molecular mass (Houde *et al*., 1992a; Ouellet *et al*., 1993). This group of proteins are assumed to act as cryo-protectants and start to accumulate in crown tissues of winter wheat when the temperature is about 17°C (Kosová *et al*., 2012; Sarhan *et al*., 1997). They represent perfect markers for LT tolerance within the Poaceae (Houde *et al*., 1992b).

Binding of transcription factors to a specific DNA element can be assessed using various methods. The most common method is the gel retardation assay, also known as the Electrophoretic Mobility Shift Assay (EMSA; Hendrickson, 1985), which can be used for both quantitative and qualitative detection of protein-DNA interaction (Hellman & Fried, 2007). The EMSA technique is often used to study the interaction between a transcription factor and its target sequence and is based on the simple assumption that a protein-DNA complex will move

slower than the free DNA probe when subjected to non-denaturing polyacrylamide gel electrophoresis. Thus, DNA/protein interaction is demonstrated by the "retarded shift" in the migration of DNA on the gel (Fig. 2.9) Labeled (radioactive or chemiluminescent) linear DNA fragments carrying the protein binding site are typically used in this assay and shifts in the gel can be visualized either through radioactive or secondary detection methods.

The efficiency of protein binding to a specific DNA sequence can also be determined using Surface Plasmon Resonance (SPR) technology (Brockman *et al*., 1999; Jönsson *et al*., 1991; Pattnaik, 2005). SPR based instruments help in visualizing and understanding the real time interactions between two biomolecules such as DNA-DNA, DNA-protein, protein-lipid, etc. without the requirement of specific labeling. The method is based on determination of the refractive index of a very thin layer of material adsorbed onto the surface of a metal film called a plasmon.

A slight change in the refractive index leads to a change in the signal generated through SPR which allows a precise measurement of interactions between two molecules. A light source is used to generate an incident light that illuminates the plasmon which is composed of a thin gold-coated prism. Reflection of the light is then recorded by a photodetector. Because all proteins have almost identical refractive index for protein adsorption it is easy to convert the refractive index between the adsorbate and the buffer into thickness and mass of the adsorbate (Pattnaik, 2005; Fig. 2.10). The SPR technique can be used to quantify the binding capacity, association and dissociation factors of DNA binding proteins to their DNA target motifs. An SPR adsorption profiling can be created by measuring the change in the angle of incidence light in respect to time due to absorbance of biomolecules on the prism surface (Green *et al*., 2000). The rate equation is expressed as:

$$d[AB]/dt = K_a[A][B]-K_d[AB] \quad \text{i}$$

Where, K_a is the association constant, K_d is the dissociation constant, [A] is analyte concentration, [B] is free ligand concentration and [AB] is the concentration of the analyte-ligand complex (Green *et al*., 2000). The maximum value of changes in the refractive index (R_{max}) is proportional to the total ligand concentration ([B]+[AB]). On the other hand the

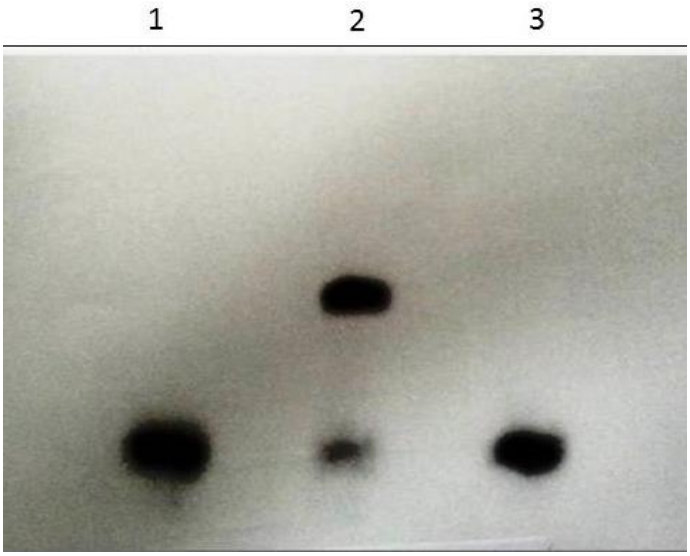

Fig. 2.9. Diagrammatic representation of EMSA. Autoradiogram of an EMSA assay performed with EBNA protein and developed by chemiluminescence. Lanes #1, EBNA-Biotin control DNA; 2, EBNA-Biotin control DNA with EBNA extract; 3, EBNA-Biotin control DNA with EBNA extract and unlabeled DNA. DNA/protein incubation was performed at 23°C for 15 min before loading on a pre-run 6% non-denaturing polyacrylamide gel and run for 45 min in 1X Tris-glycine-EDTA buffer. DNA-protein complexes were then transferred to nitrocellulose membrane by electro-transfer and complexes were developed by using a Lightshift Chemiluminescent EMSA kit (Life technologies, Burlington, ON, Canada) and were visualized using a gel documentation system (GelDoc XR+, Bio-Rad, Hercules, CA, USA).

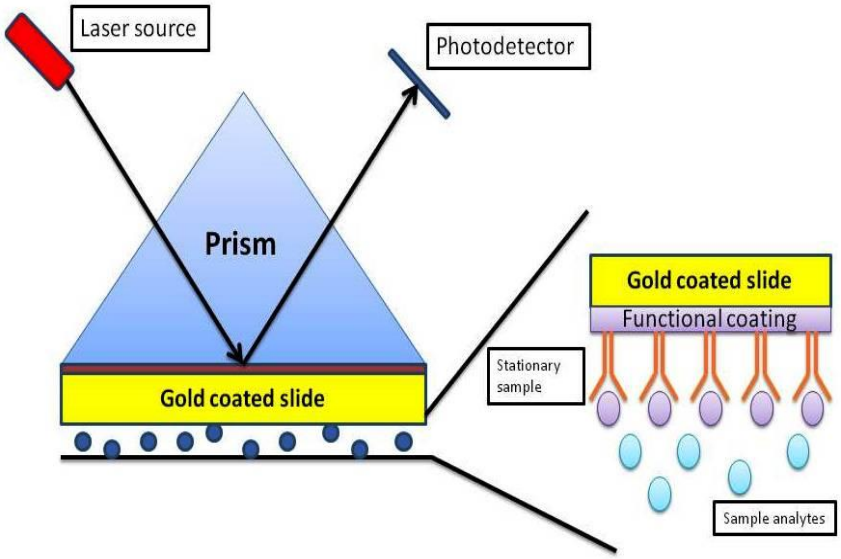

Fig. 2.10. Schematic illustration of SPR assay. One of the putative interacting samples is bound to a surface (a commonly used surface is carboxymethylated dextran) and then a flow of the other sample is passed over the bound molecules. A laser light source is used to obtain the refractive index which is detected by a photodetector. Any interaction between the two molecules will generate a change in the SPR signal (modified from www.lamdagen.com).

concentration of [A] is constant as it is continuously replenished under constant flow condition. Hence, the above equation can be re-written as,

$$dR/dT = K_aC(R_{max}-R)-K_dR$$

$$= K_aCR_{max}-(K_aC+K_d)R \quad \text{..... ii}$$

From equation ii, rate constants (K_a and K_d) are calculated by plotting dR/dT vs R and affinity can be calculated as K_a/K_d (Green *et al*., 2000). The data thus obtained are represented as the on rate and off rate of the interaction between two macromolecules and is determined by the rate of SPR angle change during this process (Cheng *et al*., 1987).

2.14. Functional properties of CBFs from winter wheat *cv.* Norstar

A recent study of 15 Norstar CBF proteins encoded from *Fr-A2* and over-expressed in *E. coli* as TrxHisS-tagged recombinant proteins, revealed a large variation in protein stability and functional properties (Jain, 2013). The TrxHisS-CBF15, -CBF17 and CBF12 were found to be exceptionally stable over a wide range of temperatures and denaturing conditions. The three recombinant CBFs also interacted strongly with the target CRT motif of the COR genes *in vitro*. Interestingly, cold or denaturing conditions were found to alter the conformation of certain CBFs which impacted their functional properties. Five of these fifteen TrxHisS-CBF proteins were found to be labile in purified condition, but stable in total cell extracts; whereas, the rest were found to be stable over a wide range of temperature or denaturing treatments. Some of these recombinant CBF proteins could only be purified in denaturing condition using 6 (M) urea in the extraction buffer. In some cases, the C-terminal signature motif, as well as the remaining C-terminal region was not necessary for the binding abilities of the TrxHisS-CBFs, suggesting variation in their structural moiety and function. Some of the recombinant CBFs showed better DNA binding capacity when exposed to cold temperature, explaining the higher activity of the CBFs under cold acclimated conditions. Based on these results the hypothesis of the current project is that there is variation exists in the structure of CBF coded by the group 5 chromosomes. In this study in addition to CBF from 5A, those from homoeologous regions on 5B and 5D will also be produced in vitro and analyzed for their structure.

CHAPTER 3

MATERIALS AND METHODS

3.1. Isolation of BAC clones

Plasmid clones of a bacterial artificial chromosome (BAC) library constructed from wheat cultivar Norstar genomic DNA (Ratnayaka *et al*., 2005) were used as source of CBF gene fragments. The BAC DNA was extracted from over-night grown *E. coli* cells, cultured at 37°C, using Sigma Phaseprep BAC DNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA).

3.2. Plasmid amplification and protein expression system

The chemically competent K12 Novablue strain (*endA1 hsdR17*($r_{k12}^{-}m_{k12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[$proA^{+}B^{+}$ *lacI qZΔM15*::*Tn10*]) was used for initial cloning of expression vectors (EMD Millipore, Darmstadt, Germany). For expression studies, the type B *E. coli* strain BLR(DE3)pLysS (*F*$^{-}$*ompT hsdSB*($rB^{-}mB^{-}$) *gal dcm* (DE3) Δ(*srl-recA*) *306*::*Tn10 pLysS*) was used (EMD Millipore, Darmstadt, Germany). Because of lower intrinsic protease activity, type B strains are better suited for production of recombinant proteins than K12 strains.

3.3. Amplification of the CBF genes

The individual CBF gene fragments were generated by PCR amplification of BAC plasmids using gene-specific oligonucleotides as primers. The forward PCR primer was designed with adaptor sequence GACGACGACAAG followed by 15-19 nucleotides corresponding to the first coding sequence of the specific CBF gene. The reverse primers had the GAGGAGAAGCCCGGT adaptor sequence followed by 21-30 nucleotides of the last CBF coding sequence including the translational stop codon. PCR amplification was performed using a DNA thermo-cycler (Model# 5345, Eppendorf, Mississauga, ON, Canada). The total volume of each reaction was 25 µL, containing 2.5 µl 10 X PCR buffer (Fermentas/Thermo Fisher Scientific, Waltham, USA), 1 X CES (2.7 M betaine, 6.7 M DTT, 6.7 % w/v DMSO, 55 µg mL^{-1} BSA), 2.5 mM $MgCl_2$, 2 mM dNTPs, 1 µM each of the forward and reverse primers, 50 ng BAC DNA and 1U *Pfu* polymerase (Fermentas/ Thermo Fisher Scientific, Burlington, ON, Canada). The PCR amplifications were performed by an initial denaturation at 94°C for 2 min

followed by 32 cycles of 94°C for 45 s, 64°C for 30 s, and 72°C for 90 s. A final extension at 72°C for 10 min was performed after completion of the cycles.

PCR reactions were analyzed on 1% w/v agarose gel and electrophoresis was performed with 1 X TAE buffer (40 mM Tris-HCl, pH 7.6, 20 mM acetic acid, 1 mM EDTA) containing 0.25% v/v ethidium bromide. The DNA fragments were visualized under low UV using a gel documentation system with an attached CCD camera (GelDoc XR+, Bio-Rad, Hercules, CA, USA). Amplified CBF products were excised from the gel, purified using Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and quantified by using a DU730 UV-Vis spectrophotometer (Beckman Coulter, Pasadena, CA, USA).

3.4. Preparation of TrxHisS-CBF expression vector

A ligation independent cloning strategy was used for cloning of the CBF amplicons using a pET32 enterokinase/ligation independent cloning vector pET-32 EK/LIC (Novagen/ EMD Millipore, Darmstadt, Germany). The specific design of the PCR primers allowed generation of 10-12 nucleotide overhangs at both the ends of the PCR products. The compatible ends in the PCR fragments for the EK/LIC vector were generated by incubating 0.2 pmol PCR generated products in a 20 µL reaction volume containing 0.4 µL T4 DNA polymerase (New England Biolabs, Whitby, Canada), 2 µL 10 X T4 DNA polymerase buffer, 2.5 mM dATP and 5 mM DTT. The reaction mixtures were incubated at 22°C for 30 min so that the T4 DNA pol could exogenously excise the nucleotide overhangs from both end of the PCR generated fragments, until an adenine residue was found on either side of the PCR fragments, which then could be reattached using the supplied dATP in the reaction mixture by the T4 DNA pol itself. T4 DNA pol enzyme was then heat-inactivated by incubating the reaction mixture at 75°C for 20 min. 0.02 pmol of these T4 DNA pol treated PCR products (insert) with compatible ends were annealed to 0.01 pmol linearized pET32EK/LIC vector (Novagen/ EMD Millipore, Darmstadt, Germany) by incubating them together at 22°C for 5 min in presence of 25 mM EDTA.

3.5. Transformation of constructs into *E. coli* BLR(DE3)pLysS cells

Expression vectors were initially transformed into *endA*$^{-}$ Novablue Gigasingles competent cells (Novagen/ EMD Millipore, Darmstadt, Germany) as described in protocol provided by the supplier. Transformants were selected on LB agar media (1% w/v bacto-

tryptone, 0.5% w/v yeast extract, 0.17 M NaCl, 1.5% w/v agar) containing 40 µg mL^{-1} carbenicillin and 12.5 µg mL^{-1} tetracycline. For protein expression, validated expression vectors were transformed into BLR(DE3)/pLys cells and selected on solid Super Broth (SB) media (2.5 % w/v bacto-tryptone, 1.5 % w/v yeast extract, 0.34 M NaCl, 1.5% w/v agar) containing 40 µg mL^{-1} carbenicicillin, 34 µg mL^{-1} chloramphenicol and 12.5 µg mL^{-1} tetracycline.

3.6. Isolation of plasmids and DNA sequencing

Plasmids were purified from *E. coli* cultures using Hi-speed plasmid mini kit (FroggaBio, North York, Canada), following the protocols supplied by the manufacturer. DNA sequence analysis of the pET32LIC/EK vector insert was performed using a T7 promoter primer (5'TAATACGACTCACTATA3') as the forward primer and T7 terminator primer (5'GCTAGTTATTGCTCAGCGG3') as the reverse primer. Sequencing was done by Robarts Research Institute, London, Ontario and data generated were analyzed using SeqMan module of Lasergene 8 software (DNASTAR Inc., Madison, WI, USA).

3.7. Production of TrxHisS-CBF fusion proteins in *E. coli*

The BLR(DE3)pLysS *E. coli* cells harboring the recombinant plasmids were grown in SB broth containing 40 µg mL^{-1} carbenicillin, 34 µg mL^{-1} chloramphenicol and 12.5 µg mL^{-1} tetracycline. Protein production was initiated by inoculating 250 mL media with a 10 mL overnight culture and growing cells at 28°C until A_{600nm} reached 0.3. IPTG was added to the cultures to a final concentration of 0.5 mM to induce production of recombinant proteins. After three or 12 h of induction, cells were harvested by centrifugation at 9,000 x g, 20 min, 4°C and cell pellets were stored at -20°C until further use.

Cells were cultured at five different temperatures according to the requirements of the experiment. For cells grown at 28°C and 37°C incubators with shakers were used. However, for cells grown at 23°C and 18°C, cooling water bath with rotatory shakers were used. To grow cells at 4°C, a rotary shaker was kept in a cold room where the temperature was maintained at 4°C.

3.8. Preparation of native TrxHisS-CBF protein extracts from *E. coli*

Three different protein extraction protocols were used for production of native soluble extracts of *E. coli*.

3.8.1. Extraction method 1

This method involved use of Bugbuster protein extraction reagent (Novagen, Billerica, MA, USA) to solubilize *E. coli* cell pellets. In this procedure, cell pellets obtained from a 50 mL culture were dissolved in four mL of Bugbuster, incubated at room temperature (RT) with continuous shaking for 20 min, centrifuged at 15,000 x g for 20 min at 4°C to generate soluble *E. coli* extract as supernatant.

3.8.2. Extraction method 2

This protein extraction method involved use of NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0; Qiagen, Toronto, ON, Canada). A 1.4 mL aliquot of the NPI-10 buffer fortified with 0.5 U benzonase nuclease (Sigma-Aldrich, St. Louis, USA), 2.5 % lysozyme and 50 µL protease inhibitor cocktail (Sigma-Aldrich) was used to dissolve cell pellets obtained from 50 mL liquid culture. The suspension was incubated on ice for 20 min and then centrifuged at 15,000 x g, 4°C for 20 min to generate the soluble extract.

3.8.3. Extraction method 3

The third protocol involved mechanical disruption of bacterial cells using a cell homogenizer (Emulsiflex C3, Avestin Inc., Ottawa, ON, Canada). In this process, cell pellets obtained from 200 mL culture were re-suspended in 40 mL of NPI-10 buffer with 0.5 U benzonase nuclease (Sigma-Aldrich) and 4 mL 1 X protease inhibitor cocktail (Sigma-Aldrich). This cell suspension was applied to ice-chilled chamber of the homogenizer and passed through the chamber under 15,000 psi air pressure for at least two min or until the cell suspension became translucent (not more than five minutes). The acquired cell lysate was centrifuged at 15,000 x g, 4°C for 20 min and the supernatant was collected as soluble protein source.

3.9. Preparation of denatured TrxHisS-CBF protein extracts from *E. coli*

A fourth protocol was used to extract fusion proteins from *E. coli* cells under denaturing conditions. In this extraction method, a buffer with urea (7 M urea, 100 mM NaH_2PO_4, 100 mM Tris-HCl, pH 8.0) was used. The cell pellet from a 50 mL culture was re-suspended in 0.7 mL of this buffer and incubated at room temperature for 20 min with continuous shaking, followed by

centrifugation at 15,000 x g at room temperature for 20 min, thereafter collecting the supernatant as the source of denatured proteins.

3.10. Purification of TrxHisS-CBF proteins

Both manual and automated purification of TrxHisS-CBF proteins were performed. Affinity chromatography was performed using 1 mL His-TrapFF column (GE Healthcare, Uppsala, Sweden). To further purify the eluted protein, fractions were subjected to anion exchange chromatography using 1 mL Hi-Trap anion exchange (GE Healthcare, Uppsala, Sweden) column.

For manual affinity purification, the Bugbuster extract (4 mL) or NPI-10 extract (1.4 mL) were applied onto the column, which was then washed with 10 mL bind buffer (0.3 M sodium chloride, 50 mM sodium phosphate buffer, 10 mM imidazole, pH 8.0). Thereafter, the column was washed with 6 mL wash buffer (0.3 M sodium chloride, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0) and proteins bound to a Ni^{+} column were eluted with 2 mL elution buffer (0.3 M sodium chloride, 50 mM sodium phosphate buffer, 250 mM imidazole, pH 8.0). Purification of proteins from extracts produced under denaturing conditions was done by applying 0.7 mL extract onto the column, followed by washes and elution as described above.

For automated protein purification, a fast liquid chromatography based protein purification system (ÄktaPure 25 protein purification system, GE Healthcare, Mississauga, Canada) was used. Soluble protein samples of one mL, ten mL or up to 50 mL were applied and purified by affinity chromatography using the Unicorn software version 6.3 (GE Healthcare, Mississauga, Canada) provided with the instrument. Protein was eluted from the column by applying a linear gradient of imidazole from 0 to 250 mM in elution buffer. Samples (25 ul) of the eluted fractions were analyzed by SDS-PAGE to identify fraction(s) to be pooled for desalting.

3.11. Anion exchange chromatography

For anion exchange, a maximum volume of 10 mL could be purified. In all the cases a sample or buffer flow of 1 mL min^{-1} was maintained. Unfortunately the majority of the purified proteins were lost during anion exchange (data not shown), which made it difficult for further

use of the proteins. Gel filtration chromatography could be used as a substitute to remove the contaminants from the purified protein fractions obtained through affinity chromatography.

3.12. Quantification of purified TrxHisS-CBF fusion proteins

The concentration of the various purified fractions collected was determined by using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Mississauga, Canada) as described by the supplier. In brief, a linear gradient concentration of bovine serum albumin (BSA) was prepared and A_{595nm} was measured using a DU730 UV-Vis spectrophotometer (Beckman Coulter, Pasadena, CA, USA) to prepare the standard curve. The concentrations of the purified recombinant CBF proteins were calculated based on this standard curve.

To calculate the actual concentration of the target TrxHisS-CBF fusion proteins present in each purified fractions, an S-tag based assay was performed. An S-tag System kit (Novagen/EMD Millipore, Billerica, MA, USA) was used for this experiment and the protocols were followed as described therein. This assay was based on the interaction between S-tag peptide (15 amino acid long pancreatic RNAase A; Kim & Raines, 1993) present at the amino terminal of the recombinant CBF proteins with ribonuclease S protein (Richards & Wyckoff, 1971). The S-tag sequence and the S-protein form an active ribonuclease that reacts with the poly(C) substrate already added to the reaction mixture, to produce soluble nucleotides. Concentration of the free soluble nucleotides could be determined by spectrophotometric methods. The concentration (pmol μl^{-1}) of S-Tag recombinant protein was calculated using the formula mentioned in the user manual supplied with the kit.

3.13. Desalting of purified proteins

Desalting of purified TrxHisS-CBF proteins was necessary to remove excess salt and imidazole from the solution and was done by dialysis against two liter 20 mM sodium chloride and 50 mM sodium phosphate buffer, pH 8.0 with three buffer changes. The spectrapor dialysis tubings made up of cellulose ester (Spectrum medical industries, Los Angeles, CA, USA) used for this process had a cut off molecular mass of 20 kDa which was suitable to dialyze the TrxHisS-CBFs, as their molecular mass ranged from 40 kDa to 70 kDa. 3.5-4 mL of purified protein was desalted and the final volume of the desalted protein was obtained at ~5 mL. The first buffer change used to be performed after an hour of dialysis and then after two more hours

the second buffer exchange used to be done. Finally an overnight dialysis used to be performed against the above mentioned buffer. The dialysis set up and procedure were maintained at 4°C.

Alternatively, 3.5-4 mL of purified protein fractions were applied onto a 5 mL HiTrap desalting column (GE Healthcare, Mississauga, Canada) and eluted against 5 mL 20 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0 at a flow rate of 5 mL min^{-1}. Glycerol was added to the desalted protein preparations to a final concentration of 10% (v/v) and the samples were subsequently flash frozen in liquid nitrogen prior to storing at -80°C temperature until further analyses.

3.14. SDS-PAGE analysis and determination of the TrxHisS-CBF protein molecular mass

Samples of fractions obtained during the purification procedure were analyzed by SDS-PAGE using gel composed of a 5 % polyacrylamide : bisacrylamide (30:0.8) stacking gel and 12 % (30:0.8) resolving gel. Samples were boiled for 4 min in the presence of 1 X SDS gel loading buffer (50 mM Tris-HCl, pH 6.8, 2 % w/v SDS, 10 % v/v glycerol, 5% v/v β-mercaptoethanol) before loading on gel. Electrophoresis was performed at constant 10 mA for 14 h using 12 X 19 cm gels and a GibcoBRL vertical gel electrophoresis apparatus. Gel was stained with Coomassie Brilliant Blue and then de-stained using 5% acetic acid solution as described elsewhere (Meyer & Lambert, 1965).

To determine the molecular mass of the fusion proteins a standard logarithmic curve was prepared based on a pre-stained protein standard (BLUeye pre-stained protein ladder, Genedirex, Froggabio, North York, ON, Canada), where the X-axis was used to plot relative distance travelled by the standard proteins and Y-axis was used to represent the logarithm of molecular masses of the same. Distance travelled by the proteins of interest on the same gel was extrapolated on this curve and their molecular mass was calculated following the formula $y = mx+b$, and solving for y as the molecular mass for the unknown proteins.

3.15. Bioinformatics analyses of the CBF proteins

The bioinformatics analyses were performed using some of the tools available at the ExPASy proteomic website (www.expasy.org/tools; Gasteiger *et al*., 2005). AGADIR and APSSP software were used to calculate the percentages of helices, β-turn and β-sheets (Yi &

Lander, 1993). ePESTfind software was used to predict the instability index and PEST scores based on the presence of stretches of proline (P), glutamic acid (E), serine (S) and Threonine (T), used as a proof of instability of the proteins (Rechsteiner & Rogers, 1996). The percentage of aromatic amino acids was calculated by using ProtParam software. Amino acid sequence homology was determined by using the online available software COBALT (Dereeper *et al.*, 2010) and T-Coffee (Di-Tommaso *et al*., 2011). www.phylogeny.fr website was used to create a phylogenetic tree on the basis of the AP2 domain and the flanking CMIII-3 and CMIII-1 amino acid sequences for the CBFs studied (Dereeper *et al.*, 2008).

3.16. End labeling of *Wcs120* DNA probe

For electrophoretic mobility shift assays (EMSA), *Wcs120* promoter DNA sequences were obtained from Sigma Genosys (Sigma-Aldrich, Oakville, ON, Canada) as artificially synthesized single stranded 21-bp complimentary oligonucleotide primer pairs. The *wcs120* probe fragments carried one CRT motif (CCGAC) that corresponds to the 677-697 nucleotides of wheat *cv*. Fredrick promoter region (5′GCCACCTGCCGACCACTGATC3′; Accession# AF031235; Vazquez-Tello *et al*., 1998). Double stranded DNA was synthesized by mixing equimolar concentrations of both the single stranded complimentary oligonucleotides in 10 mM Tris-Cl buffer, pH 7.5, heating them at 95°C for four minutes and then gradually decreasing the temperature to 23°C over a period of 45 min using a thermo-cycler (Model# 5345, Eppendorf, Mississauga, Canada). This double stranded DNA probe was radiolabeled in a 20 µL reaction mixture containing 2 µL 10 X kinase buffer (Invitrogen, Carlsbad, CA, USA), 10 pmol of [γ-^{32}P]ATP (6000 Ci/mmol, Perkin Elmer, Waltham, MA, USA), 50 nmol of 21-bp *wcs120* DNA fragment and one unit of T4 DNA polynucleotide kinase (Invitrogen, Carlsbad, CA, USA). The labeling reaction was incubated at 37°C for an hour, terminated by adding 4 µL of 250 mM EDTA and purified using Illustra™ ProbeQuent G-50 microcolumns (GE Healthcare, Buckinghamshire, UK) following the supplied manufacturer's protocol. Incorporated isotopes were quantified using a Bioscan Quickcount radioisotope counter (Model QC-4000XER, Highland Scientific, Washington DC, USA).

For surface plasmon resonance (SPR) experiments, the identical *Wcs120* promoter DNA sequence as complimentary single stranded oligonucleotides were obtained from Eurofins Genomics (Huntsville, AL, USA) with a specific biotin tag (-BioTegQ) attached to the 3′ end.

Double stranded DNA fragments were generated as described above and used for the SPR experiments.

3.17. Electrophoretic mobility shift assay (EMSA)

Purified TrxHisS-CBF proteins (section 3.10) were diluted in 10 mM Tris-Cl, pH 7.5 for the EMSA assay. The 20 µL reaction mixtures were prepared by mixing 10 mM Tris-Cl, pH 7.5, 0.1% Triton-X100, 5% glycerol, 20 mM DTT, 2.8 µg sheared salmon sperm DNA with [γ-^{32}P] end-labeled *wcs120* DNA probe (~5,000 cpm) and 1 µg of each of the purified TrxHisS-CBF proteins. Reactions were incubated either at 4°C or at room temperature for 20 min or an hour. Samples were loaded directly on a pre-run (200 volts, 30 min) 6% 30:0.8 acrylamide:bisacryl non-denaturing polyacrylamide gel along with 1 X gel loading buffer (0.125% w/v bromophenol blue, 0.125% w/v xylene cyanol and 20% w/v sucrose) on one lane to track the progress of the samples on gel. The electrophoresis was performed in 1 X Tris-glycine-EDTA buffer (25 mM Tris, 0.19 M Glycine, 1 mM EDTA) for two hours at 200 volts using a vertical gel electrophoresis unit (Model# V16-2, Life technologies, Carlsbad, CA, USA). Gels were then dried for 45 min at 85°C using a gel dryer (Model# 583, BioRad, Hercules, CA, USA), exposed to Kodak X-ray films (Eastman Kodak, Rochester, NY, USA) at -80°C for 15 h and visualized using a gel documentation system with attached CCD camera (GelDoc XR+, BioRad, Hercules, CA, USA). Experiments were performed in duplicates.

3.18. Surface plasmon resonance (SPR) experiment

Purified TrxHisS-CBF proteins (section 3.10) were diluted in phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5) with 0.1% v/v Tween-20. A Bio-Rad ProteOnXPR36 system was used for the assay. A neutravidin coated gold chip (Bio-Rad, Hercules, CA, USA) was used to study the DNA-protein interaction. The chip was conditioned and regenerated by flowing 1 M NaCl + 50 mM NaOH over it at a flow rate of 30 µL min^{-1} for 1 min in both horizontal and vertical directions. Various concentrations of the biotinylated *wcs121* probe fragment were immobilized to the chip in five different channels, ranging from 20 to 1.25 nM in serial dilutions. Flow rate was maintained at 50 µL min^{-1} for 30 s. The DNA-protein interaction was observed by passing five different concentrations of the dialyzed TrxHisS-CBF proteins, ranging from 1.0 µM to 12.5 nM at one third serial dilutions

over the various concentrations of immobilized biotinylated probe fragments. Dissociation was performed by flowing 0.1% w/v SDS at a rate of 100 μL min^{-1} for 18 s twice over the chip surface followed by two washes with 10 mM HCl at a rate of 100 μL min^{-1} for 18 s. Any interaction between TrxHisS-CBFs and *Wcs120* promoter sequence was detected by a sensor that detects any change in the refractive index of the light and resulted in a curve clearly showing association and dissociation. The kinetic analyses including on rate (K_a), off rate (K_d) and affinity (K_D) of the interaction were calculated by using the ProteOn™ manager software provided, based on a simple 1:1 Langmuir kinetic equation (Langmuir, 1918). All experiments were performed at 23°C constant temperature and in duplicates.

CHAPTER 4

RESULTS

4.1. Amplification and cloning of CBF genes from the Norstar BAC library

A total of 13 CBF gene fragments were amplified from individual BAC clones carrying Norstar CBF genes (Table 4.1). All PCR reactions were done using *Pfu* Polymerase to assure high fidelity during the amplification process. After amplifying and purifying the CBF amplicons, the protocol yielded 0.5 to 1.9 μg of DNA that was adequate for all further experiments (Fig. 4.1; Table 4.1). The DNA fragments with PCR generated predetermined overhangs were incubated with dATP, in presence of T4 DNA polymerase to excise both the strands until an Adenine residue was present on the over hangs, where it could reattach the excised adenine residue using dATP. This formed a compatibility strand which thereafter could be annealed to the compatible ends of protein expression pET32/EK-LIC vector (described in details in section 3.4). The constructed vector was transformed into Novablue *E. coli* K12 strain for plasmid amplification. The amplified plasmid DNA was extracted and verified for correct sequence of recombinant *Cbf*s in all the vectors. Plasmids with accurate sequences were transformed into BLR(DE3)pLysS type *E. coli* cells for over-production of recombinant proteins.

4.2. Over-expression of the recombinant TrxHisS-CBF proteins in *E. coli*

By following the protocols above, 12 out of the 13 CBF genes could be successfully over-expressed in an *E. coli* system as TrxHisS- tagged fusion proteins. To maintain the protein production during the log phase of bacterial growth, early induction with IPTG was performed. Previously two hours incubation was done (Jain, 2013), but in this study an induction time of three hours was found to be optimal for production of fusion proteins in adequate quantities (Fig. 4.2). One mL cultures for each of the CBF clones were collected immediately after the addition of IPTG and then again when the total induction period was completed. The cell cultures were centrifuged at 10000 x g for four minutes at room temperature and the cell pellets thus obtained were re-suspended in 1 X SDS gel loading buffer and boiled for five minutes to obtain the total cellular protein fractions. For each of the CBF clones, total cellular proteins obtained before (-) and after (+) induction analyzed on 12% SDS-polyacrylamide gels revealed that the peptide bands corresponding to the respective TrxHisS-CBFs stained intensely after induction (Fig. 4.2).

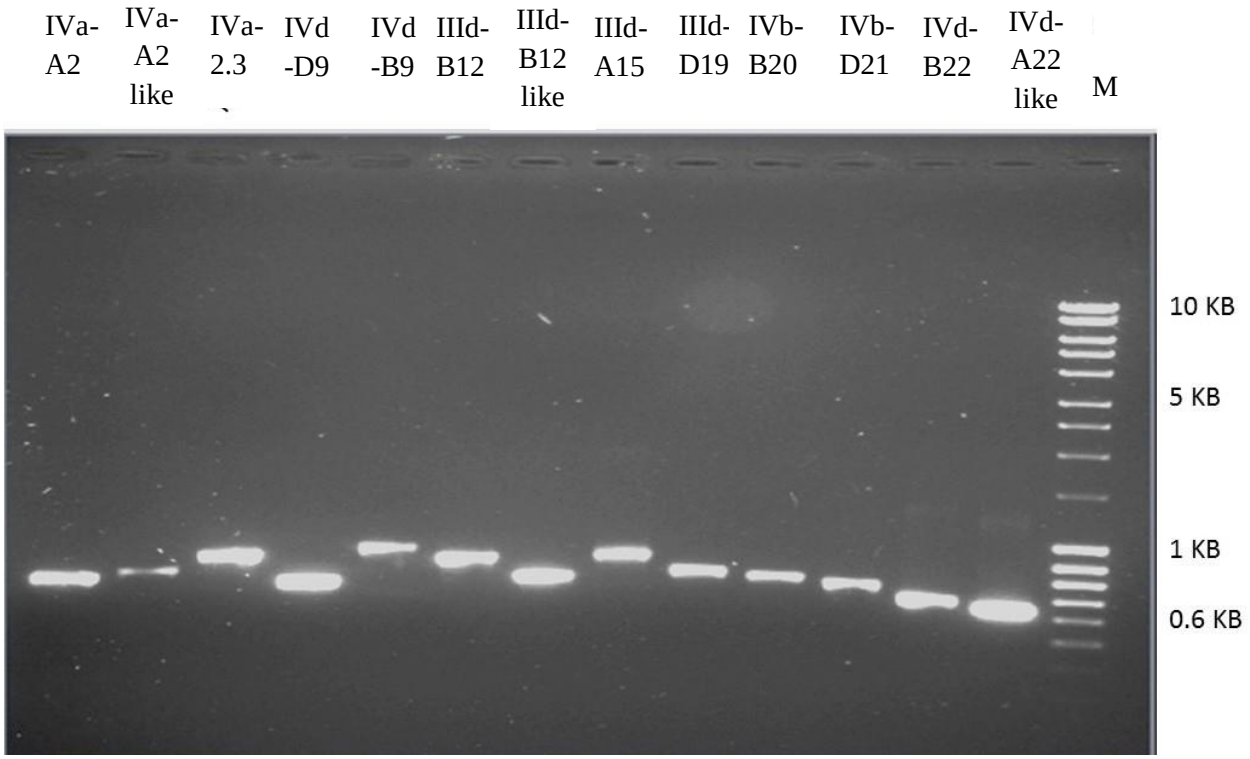

Fig. 4.1. Purified PCR fragments of 13 CBF genes from winter wheat *cv*. Norstar. Gel purified PCR products separated on 1% agarose gel electrophoresis and stained with ethidium bromide. The CBF designations are based on Badawi *et al*., 2007 (see Table 4.1). An equal volume of purified DNA was loaded per lane. Migration of DNA size standard is shown to the right.

Table 4.1 BAC clones carrying selected CBFs and their amplicons

CBF subgroup*	CBF name	BAC clone**	CBF designation***	Yield (µg)		Size of the amplicons (bp)
				BAC clone plasmid	Purified CBF amplicons	
IIId	CBF12	1558M10	CBFIIId-B12	0.5	1.2	810
	CBF12	260E13	CBFIIId-B12 like	0.6	1.0	695
	CBF15	1558M10	CBFIIId-A15	0.5	0.9	825
	CBF19	1179D19	CBFIIId-D19	0.9	1.3	715
IVa	CBF2	260E13	CBFIVa-A2	0.6	1.7	720
	CBF2	926I25	CBFIVa-A2 like	0.3	0.5	740
	CBF2.3	926I26	CBFIVa-2.3	0.3	1.0	760
IVb	CBF20	1374F6	CBFIVb-B20	0.7	0.9	695
	CBF21	1374F6	CBFIVb-D21	0.7	1.0	720
IVd	CBF9	653A22	CBFIVd-D9	0.9	1.9	635
	CBF9	1548G4	CBFIVd-B9	1.5	0.7	870
	CBF22	341J20	CBFIVd-B22	0.8	1.5	675
	CBF22	926I26	CBFIVd-A22 like	0.6	1.9	605

* CBF sub-group designations based on Mohseni *et al*., 2012

** BAC clone designations based on Ratnayaka *et al*., 2005

*** CBF designations based on Badawi *et al*., 2007

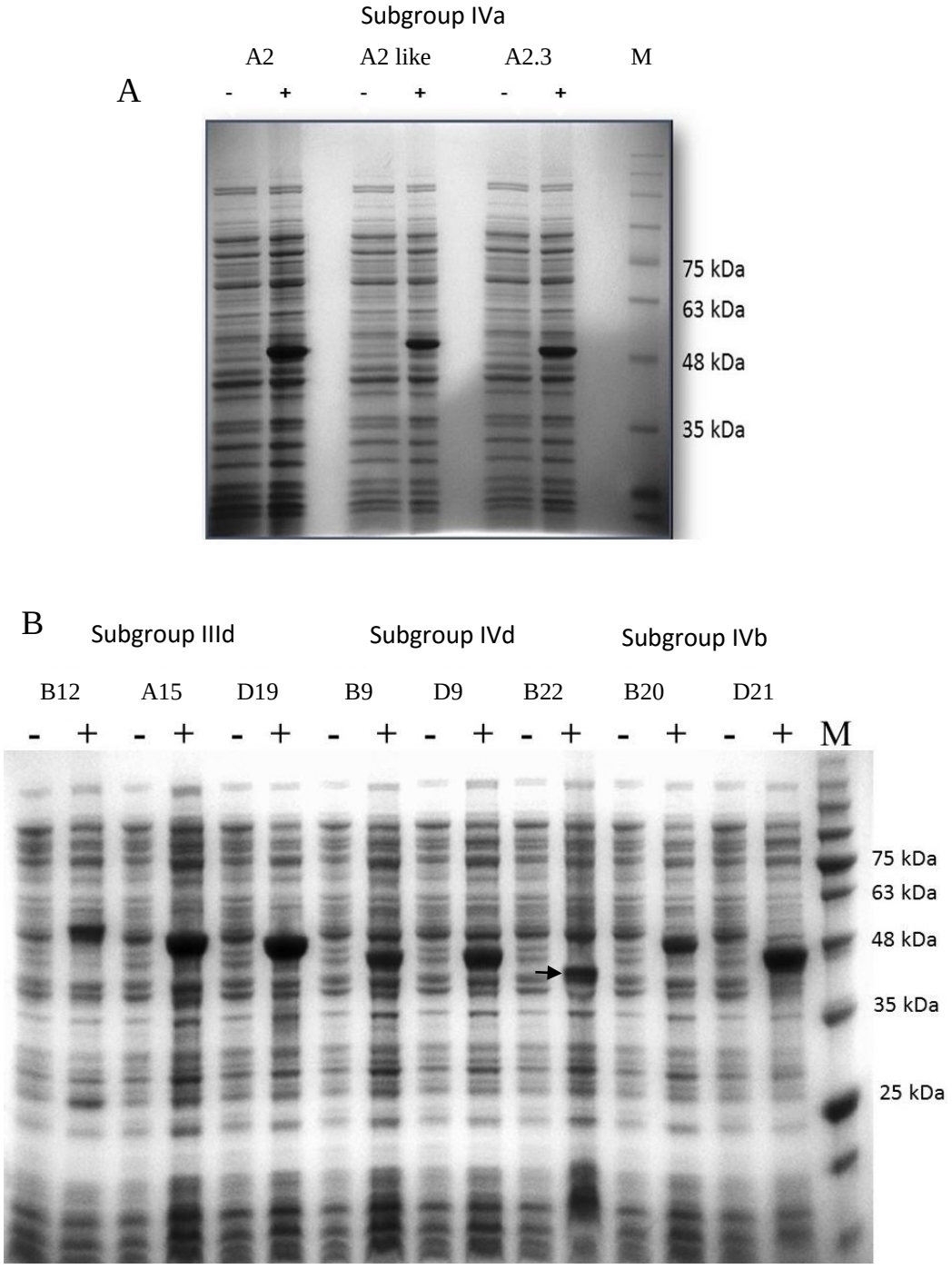

Fig. 4.2. SDS-PAGE analyses illustrating over-expression of the TrxHisS-CBFs in *E. coli* BLR(DE3)pLysS cells. Coomassie blue stained total cellular proteins before (–) and after (+) induction for three hours with IPTG. Only CBFIVd-B22 needed an overnight incubation and it is marked by an arrow (→). Lane M is protein molecular weight marker.

Eleven TrxHisS-CBF proteins could be over-expressed when incubated at A_{600nm} of 0.3 and grown for three hours thereafter. However, TrxHisS-CBFIVd-B22 failed to show any protein production even after five hours of incubation. These cells had a detectable amount of protein production only when subjected to overnight growth after induction. Thus, an overnight incubation was necessary to successfully over-express adequate amounts of TrxHisS-CBFIVd-B22 (Fig. 4.2B) which could be repeated successfully during the rest of the study.

4.3. Extraction of recombinant TrxHisS-CBF proteins

4.3.1. TrxHisS-CBF protein extraction using Bugbuster reagent

Three different methods were used to solubilize over-expressed TrxHisS-CBF fusion proteins from *E. coli* cell pellets. Six out of 12 over-expressed TrxHisS-CBF proteins were extracted using Bugbuster reagent fortified with 1 X protease inhibitor complex. Four of these were cloned during this study, i.e., TrxHisS-CBFIIId-B12, -CBFIIId-A15, -CBFIVa-A2, -CBFIVd-B9 and two recombinant CBFs were taken from a previous study (TrxHisS-CBFIIId-A12 and –CBFIIId-B15) to check the efficiency of the extraction protocol. The concentration of the total cellular proteins varied from 57.1 mg mL^{-1} in TrxHisS-CBFIIId-A15 to 73.4 mg mL^{-1} in TrxHisS-CBFIIId-B12. But, when these extracted fractions were purified using nickel affinity chromatography, a considerable amount of degradation was observed in the purified fractions (Fig. 4.3A). Both TrxHisS-CBF12 and -CBF15 isoforms showed bands corresponding to the degradation products (also observed and identified as degradation products of TrxHisS-CBFs in a previous study by Jain, 2013) and only a very small amount could be obtained as intact purified recombinant CBFs.

To check if the native protease enzymes had any role in the degradation of the recombinant CBFs, proteins were extracted using Bugbuster reagent fortified with increasing concentration of protease inhibitor cocktail (P8849; Sigma-Aldrich). Even a four-fold concentration increase of protease inhibitor cocktail above that recommended concentration did not result in any improvement of TrxHisS-CBF yields (Fig. 4.3B). This was easily visualized in the gel that even though the over-expressed protein band is present in the induced total cellular protein fraction, the corresponding band to the same protein band was very weak in the cell soluble (Sol) fraction (Fig. 4.3A). During purification several wash steps were performed prior to

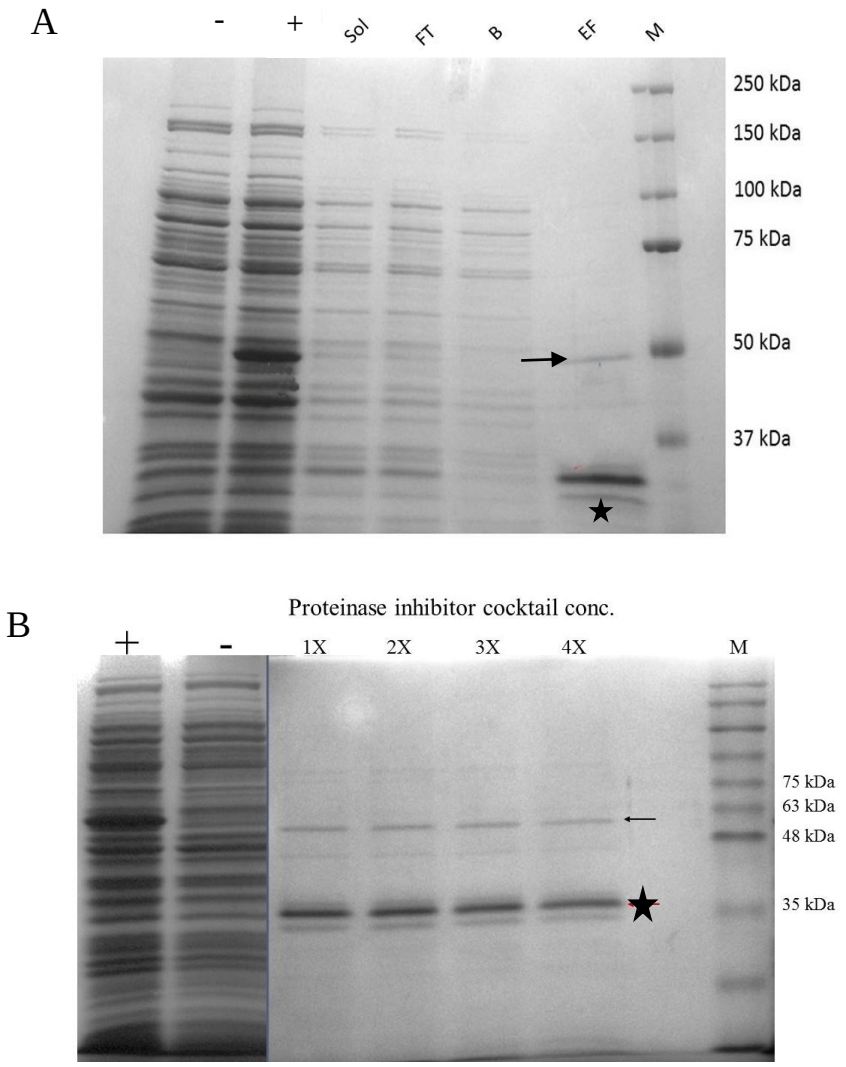

Fig. 4.3. Extraction of TrxHisS-CBFIIId-B12 fusion protein using Bugbuster reagent. A; protein extracted using Bugbuster reagent and purified using nickel affinity chromatography showing degradation of protein in the eluted fraction (EF). B; Protein extraction by Bugbuster reagent with different concentrations of protease inhibitor complex. Even in the presence of 4 X concentration of protease inhibitor complex, degradation bands could still be observed in the purified fractions. Total cellular fraction before (–) and after (+) three hours of induction period. Concentration of protease inhibitor 1X, 2X, 3X, 4X; Protein molecular weight marker lane (M). Over-induced TrxHisS-CBFIIId-B12 is marked on gel with a black arrow (→) and degraded protein bands are shown by a star (★).

eluting the protein (FT; sample collected after flowing it through the nickel column, B; protein fraction obtained after washing with bind buffer and W; protein fraction obtained after washing the column with wash buffer). However, none of the fractions contained the over expressed protein (Fig. 4.3B). In the final eluted fraction most of the proteins obtained were smaller than the expected size, suggesting degradation of the CBF fusion proteins (Fig. 4.3B). The degradation problem could not be overcome as long as Bugbuster reagent was used as the extraction medium. Most of the TrxHisS-CBFs, especially the TrxHisS-CBFIIId-D19, -CBFIVb-B20, -CBFIVb-D21 and –CBFIVd-B22 fusions were very difficult to obtain in soluble form with the Bugbuster reagent.

4.3.2. TrxHisS-CBF protein extraction using NPI-10 buffer

To evaluate a different extraction procedure, the NPI-10 buffer used in the Qiagen protein purification protocol (cat# 30725) was tested. Use of NPI-10 buffer (1.4 mL) with protease inhibitor cocktail significantly improved the extraction of cellular proteins and most of the recombinant CBF fusion proteins in soluble cell extract. The total proteins extracted, ranged from 94.5 mg mL^{-1} in TrxHisS-CBFIVd-D9 to 170.7 mg mL^{-1} in TrxHisS-CBFIVd-B9, which was more than twice the yield that was obtained using Bugbuster reagent. While purifying TrxHisS-CBFIIId-B12 from extracted soluble proteins using nickel affinity chromatography, a major polypeptide band was observed in the eluted fraction. The eluted fraction resulted in polypeptides corresponding to a similar size band in the total cell extract after induction and cell soluble fraction that was loaded on the nickel affinity chromatography column (Fig. 4.4). Following this method, TrxHisS-CBF2 and -CBF9, -CBF12 and -CBF15 isoforms could be extracted without causing degradation to the fusion proteins.

However, the improved protocol with NPI-10 buffer could not efficiently extract TrxHisS-CBFIIId-D17, -CBFIIId-D19, -CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 fusion proteins in the cell soluble extracts. To assess if the recombinant proteins were aggregated in protein inclusion bodies, the remaining insoluble cell pellets were re-suspended in 1 X SDS gel loading buffer and analyzed by SDS-PAGE. The results showed the presence of a major polypeptide band corresponding to respective recombinant CBF in the proteins extracted from cell pellets (Fig. 4.5). This suggested that these specific CBF fusion proteins were aggregating in *E. coli* and could not be obtained in the soluble form with NPI-10 buffer.

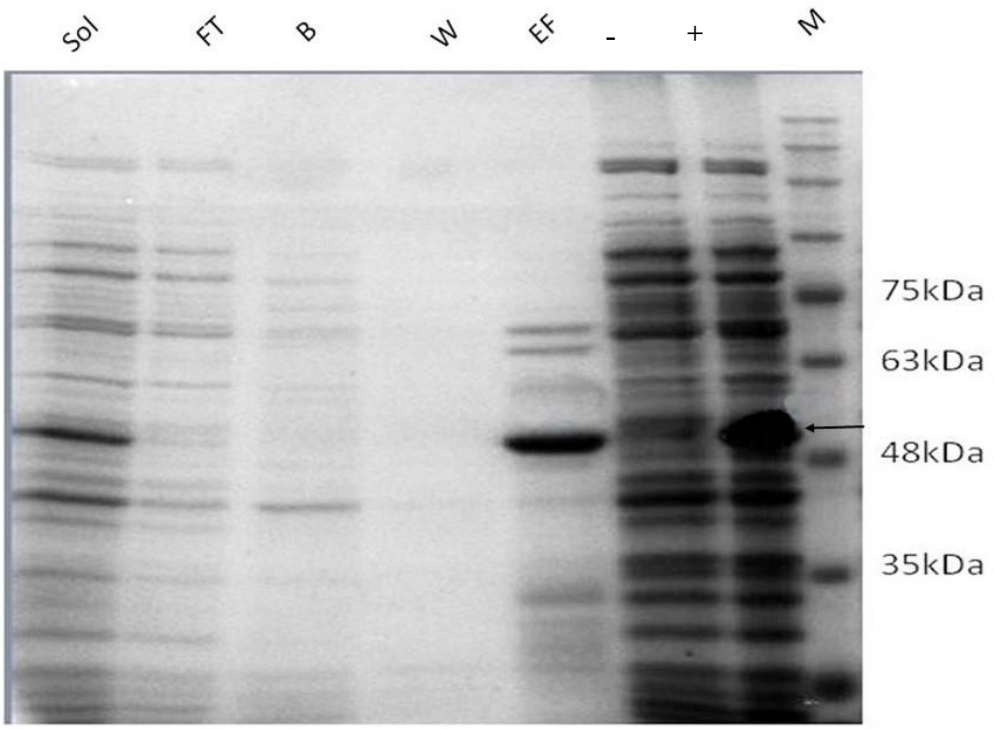

Fig. 4.4. Extraction of TrxHisS-CBFIIId-B12 protein using NPI-10 buffer followed by purification by nickel affinity chromatography. SDS-PAGE analysis on a 12% polyacrylamide gel stained with Coomassie brilliant blue of total cellular proteins extracted with NPI-10 buffer prior (─) and three hours after (+) the induction with IPTG. The other lanes show the purification steps using nickel affinity chromatography using soluble cell extract (Sol) before loading on a nickel affinity column, flow through (FT), Binding buffer fraction (B), Wash buffer (W) and eluted fraction (EF). Migration of the CBF is marked by an arrow (→) on the gel. Lane M is protein molecular weight marker.

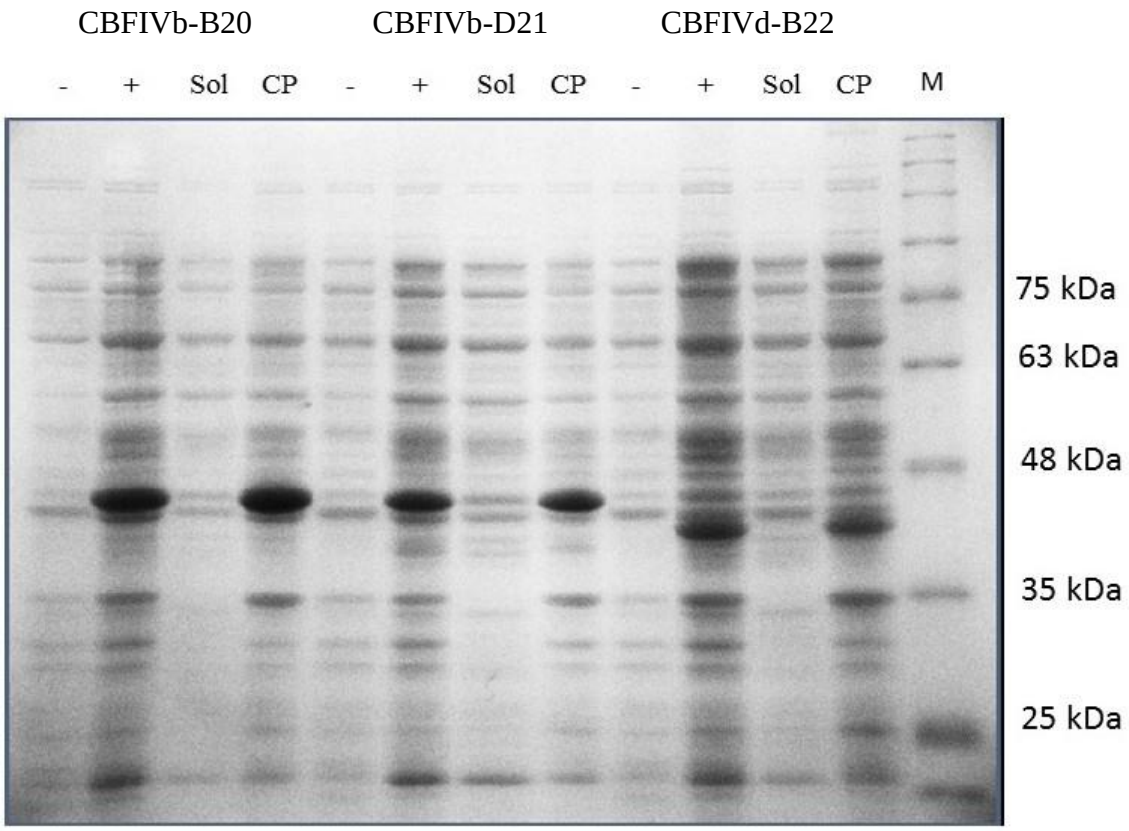

Fig. 4.5. TrxHisS-CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 formed inclusion bodies that were trapped in cell pellet. SDS-PAGE analysis on a 12% polyacrylamide gel stained with Coomassie blue of total cellular proteins before (—) and after induction (+) with IPTG that are present in the soluble (Sol) and remaining cell pellet (CP) fractions following the extraction with NPI-10 buffer. In all the cases, these recombinant CBFs aggregated to form inclusion bodies. M; protein molecular weight marker.

Several strategies to overcome the aggregation problem and increase the solubility of TrxHisS-CBFIIId-D17, -CBFIIId-D19, -CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 recombinant proteins were tested. These included, use of different growth temperatures for *E. coli* cultures, culture media with varying pH, and varying protein extraction buffers to obtain these recombinant TrxHisS-CBF proteins in their native form in cell soluble fractions.

4.3.2.1. Effect of temperature on solubility of recombinant CBFs

The A_{600nm} did not show any increase in *E. coli* cell cultures harboring the constructs of recombinant TrxHisS-CBFIIId-D17, -CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 at 4°C, suggesting that these cells were not able to grow at lower temperatures. Whereas cell cultures at 18°C, 23°C, 28°C and 37°C showed an increase at A_{600nm} as expected and cells were able to over-express the recombinant CBF proteins after addition of IPTG. SDS-PAGE analysis of total cellular proteins from *E.coli* harboring TrxHisS-CBFIVb-B20, prior and after induction with IPTG showed the presence of an induced polypeptide of corresponding size at all the four temperatures (Fig. 4.6). However, no corresponding polypeptide could be detected in the cell soluble extracts prepared with NPI-10 buffer (Fig. 4.6). Similar results were also obtained with TrxHisS-CBFIIId-D17, -CBFIVb-D21 and -CBFIVd-B22 (data not shown).

4.3.2.2. Effect of extraction buffer pH on solubility of recombinant CBFs

E. coli cells harboring one of three TrxHisS-CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 recombinant proteins were extracted with NPI-10 buffer at four pH 7.5, 8.0, 8.5 and 9.0. SDS-PAGE analyses of total cellular proteins prior to and after induction with IPTG and cell soluble phase, showed the presence of TrxHisS-CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 only in total cellular proteins (Fig. 4.7). No corresponding polypeptide could be detected in cell soluble extract. However, a polypeptide band corresponding to recombinant TrxHisS-CBFIVb-D21 could be observed in cell soluble extract with NPI-10, pH 8.0 (Fig. 4.7B). However, the yield of recombinant polypeptide was less than other recombinant CBFs that could easily be obtained in cell soluble extracts following the similar method.

4.3.3. TrxHisS-CBF Protein solubilization in denaturing form

In another strategy, urea ($CO(NH_2)_2$) was added to NPI-10 buffer to a final concentration

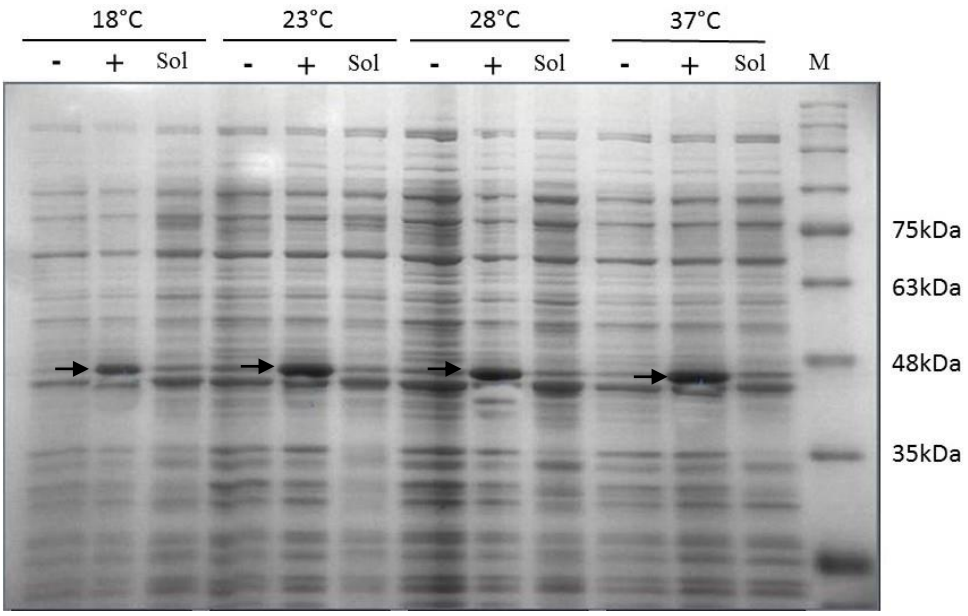

Fig. 4.6. Effect of cell culture temperature on TrxHisS-CBFIVb-B20 production. SDS-PAGE analyses of total cellular proteins on 12% polyacrylamide gel stained with Coomassie blue before (—) and after (+) 3 h of induction with IPTG and cell soluble extract (Sol) grown at four different (18, 23, 28 and 37^{o}C) temperatures. M; protein molecular weight marker. Position of the TrxHisS-CBFIVb-B20 protein on the gel is shown by an arrow (→).

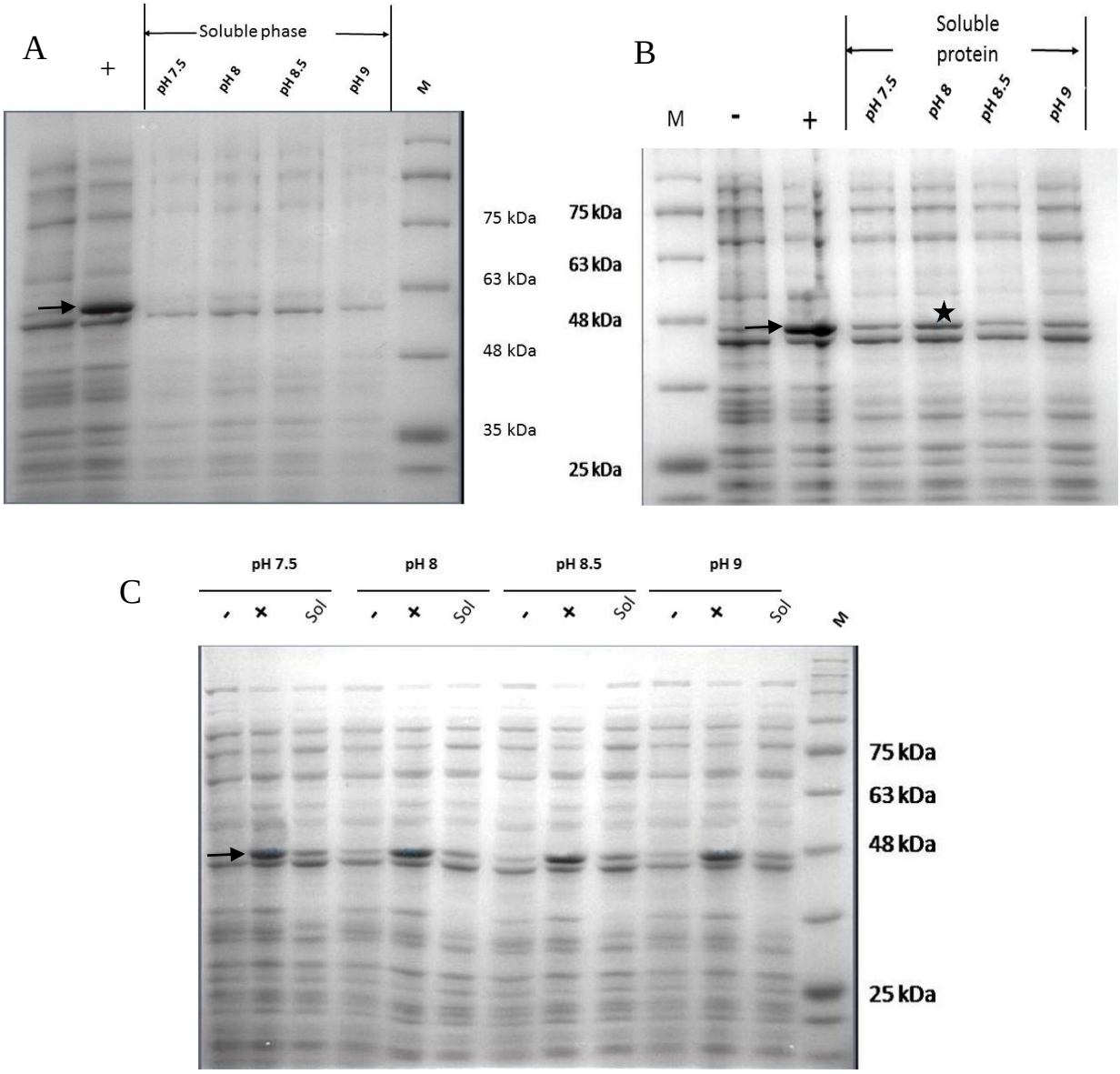

Fig. 4.7. Effect of NPI-10 extraction buffer pH on solubility of TrxHisS-CBFs produced in *E.coli*. SDS-PAGE analyses of total cellular proteins on 12% polyacrylamide gel stained with Coomassie blue before (─) and after (+) induction with IPTG and cell soluble extracts (Sol) at different pH. A; TrxHisS-CBFIVb-B20; B; TrxHisS-CBFIVb-D21 and C; TrxHisS-CBFIVd-B22. Migration of the recombinant proteins are marked on the gel by an arrow (→) and the extracted TrxHisS-CBFIVb-D21 at pH 8.0 is marked by a star (★). M; Protein molecular weight marker.

of 6 (M) and the *E.coli* cells carrying TrxHisS-CBF plasmids were re-suspended in this modified buffer, incubated at room temperature for 20 min and centrifuged at 15,000 x g for 20 min at room temperature. The supernatant was collected as source of denatured proteins in total cell extract. The total cell extract after induction was purified using nickel affinity chromatography. SDS-PAGE analyses revealed a polypeptide band corresponding to the size of TrxHisS-CBFIVb-B20 and -CBFIVb-D21 in the total cellular extract after induction as well as in soluble cell extract. Similar size polypeptide bands were also observed in the respective eluted fraction from the nickel affinity chromatography (Fig. 4.8). TrxHisS-CBFIVb-B20, -CBFIVb-D21 and -CBFIVd-B22 could be obtained and purified following this protocol. Denatured proteins can be refolded, but it is quite unlikely that the refolded protein would form the native functional form. Therefore, these proteins obtained in their denatured forms could not be suitably used for functional studies.

4.3.4. TrxHisS-CBF protein extraction by physical method

The third protocol for extracting recombinant TrxHisS-CBFs in soluble form as described in section 3.8.3 resulted in better yield of recombinant CBF protein. Following this protocol larger volumes of soluble protein could be extracted. Aliquots of 0.5 U benzonase nuclease and one mL 1 X SIGMAFAST protease inhibitor cocktail (Sigma-Aldrich) were added to 45 mL of NPI-10 buffer and this was used to dissolve frozen cell pellets obtained from 250 mL cultures. Instead of incubating the re-suspended cell pellets on ice, dissolved cell pellets were mechanically disrupted by using air pressure in an Emulsiflex cell homogenizer. The exposure pressure and time to disrupt the cells are important factors that needed to be optimized. In addition, the effect of heat generated during cell disruption was another aspect that could affect recombinant protein extraction and integrity.

4.3.4.1. Effect of exposure time to high air pressure

The time for homogenization was found to be important for extraction of soluble CBF fusion proteins. SDS-PAGE analyses of *E. coli* cells harboring TrxHisS-CBFIVd-D9 showed the presence of a corresponding polypeptide with increased intensity in the induced total cell extracts (Fig. 4.9). Homogenization for 2 or 6 min revealed a similar amount of induced peptide in the cell soluble extract. It was evident from the gel that exposing the cells for longer time under

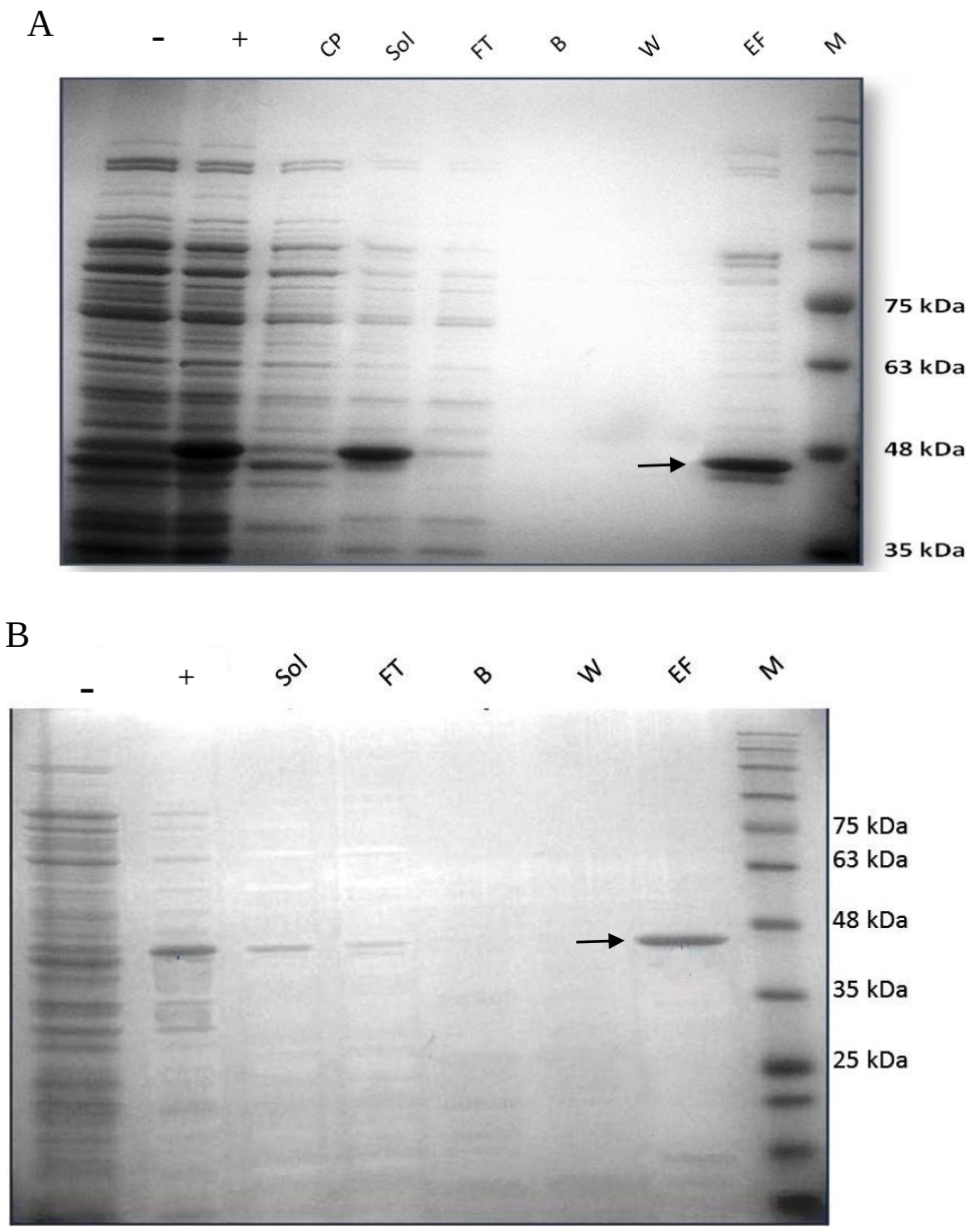

Fig. 4.8. Aggregated TrxHisS-CBF proteins extracted and purified in their denatured form. A; TrxHisS-CBFIVb-B20 and B; TrxHisS-CBFIVb-D21. This 12% polyacrylamide gel stained with Coomassie brilliant blue shows, total cellular proteins before (─) and after (+) induction for three hours, denatured soluble (Sol), total cell fraction (CP), flow through (FT), wash by binding buffer (B), sample wash buffer (W), eluted fractions (EF), and protein MW marker (M). Migration of the fusion proteins are shown on the gel by an arrow (→).

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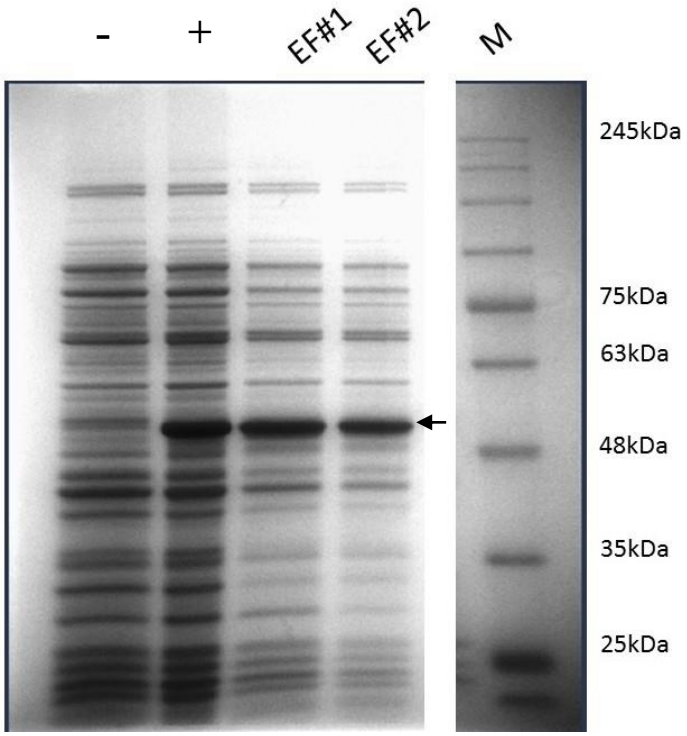

Fig. 4.9. Effect of air pressure exposure time of *E. coli* cells harboring TrxHisS-CBFIVd-D9 on extraction of recombinant proteins in cell soluble extracts. SDS-PAGE analyses of a 12% polyacrylamide gel stained with Coomassie blue of total cellular proteins before (—) and after (+) complete induction with IPTG and cell soluble extract with two (EF#1) and six (EF#2) min of high pressure exposures of cells. Lane M is protein molecular weight marker. Position of the CBF is shown on gel by an arrow (→).

air pressure did not increase the yield of the protein in the soluble fraction (Fig. 4.9). Majority of the TrxHisS-CBFs could be obtained in soluble form only within 2 min of extraction at a constant air pressure of 15,000 psi (TrxHisS-CBFIVd-B9, -CBFIIId-B12, -CBFIIId-A15, -CBFIVa-A2 like), however, some of the recombinant CBFs required an extraction time of more than 2 min (TrxHisS-CBFIIId-D17, -CBFIVb-B20, -CBFIVb-D21, -CBFIVd-B22). Depending on the CBFs, this extraction time varied from 3 to 5 min. More than 5 min to high air pressure was found to be unnecessary (Fig. 4.9). Two minutes extraction under high pressure seemed to be insufficient for TrxHisS-CBFIVb-B20, -CBFIVb-D21 and -I26-CBF22 proteins. When, *E. coli* cells harboring these recombinant CBFs were mechanically disrupted using the homogenizer for 3 min under 15,000 psi air pressure, these three recombinant CBFs were obtained in the cell soluble extract (Fig. 4.10). It was probably due to the high level of aggregation of these recombinant proteins in the cells, which required prolonged extraction time (Fig. 4.10B, C).

In the case of TrxHisS-CBFIIId-D17 extraction, a much longer exposure time (5 min) was required to obtain the protein in cell soluble extracts. Thus, when *E. coli* cells harboring recombinant TrxHisS-CBFIIId-D17 were passed through the homogenizer under 15,000 psi air pressure for 5 min, the fusion protein was obtained in cell soluble extracts (Fig. 4.10A).

4.3.4.2. Effect of heat generated during protein extraction in cell homogenizer

Heat is generated when cells are exposed to high pressure in the homogenizer chamber. . It was observed that when the homogenization time exceeded three minutes, the temperature inside the air chamber increased to ~38°C, which was significantly higher than the average room temperature of 23°C. TrxHisS-CBFIIId-D17 and -CBFs20, -21 and -22 isoforms could not be purified previously and therefore it was necessary to assess if the heating of the re-suspended solution aided protein extraction. Heat also denatures proteins and was previously shown to improve extraction of proteins in cell soluble extracts (Fu *et al*., 2013). Cell pellets containing TrxHisS-CBFIIId-D17 were re-suspended in NPI-10 and four different protocols were used to assess the efficiency of protein extraction. Two of these sets were incubated at 38°C for ten and 20 min respectively. The third cell suspension was passed through the homogenizer for 2 min at 15,000 psi air pressure and the last was passed through the homogenizer for 5 min at 15,000 psi (Fig. 4.11).

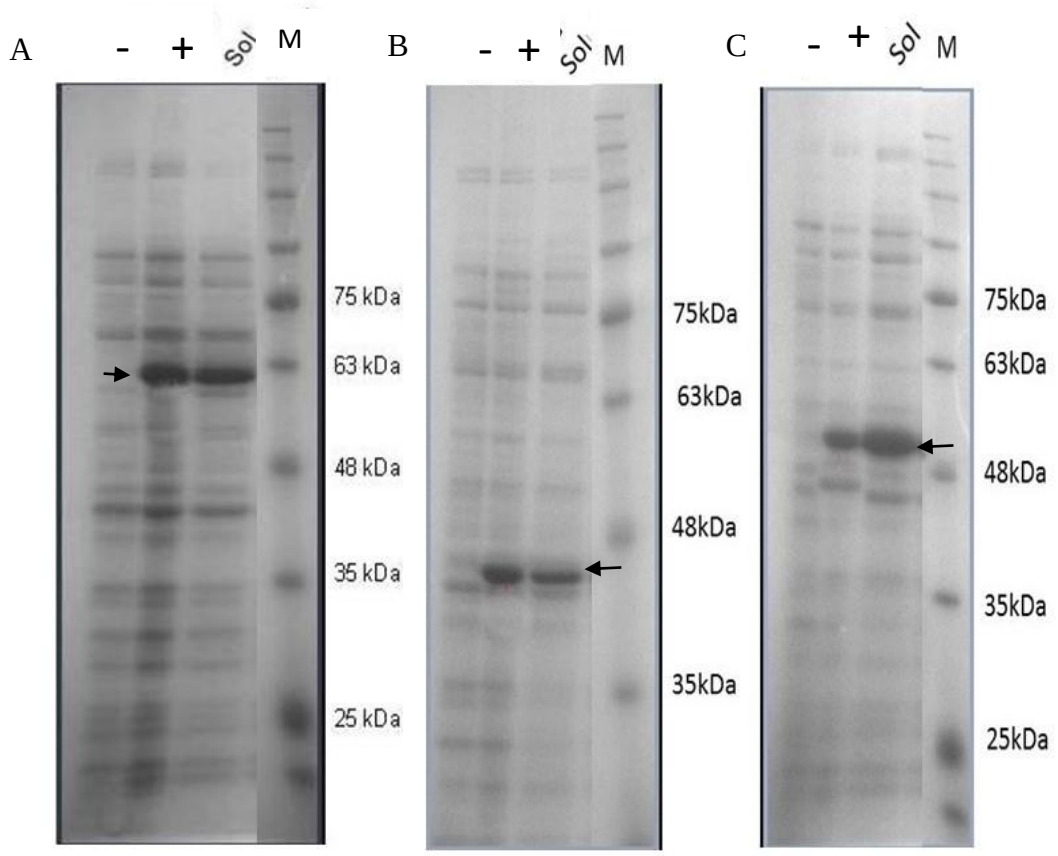

Fig. 4.10. High pressure cell disruption for 3 or 5 min releases aggregated proteins into cell soluble extracts. SDS-PAGE analyses on a 12% polyacrylamide gel stained with Coomassie blue of total cellular proteins before (–) and after (+) complete induction with IPTG and in cell soluble extracts (Sol) of cells disrupted with high pressure. A; TrxHisS-CBFIIId-D17; B; TrxHisS-CBFIVb-B20 and C; TrxHisS-CBFIVb-D21. M; Protein molecular weight marker. Migration of recombinant CBFs are marked on the gels by an arrow (→).

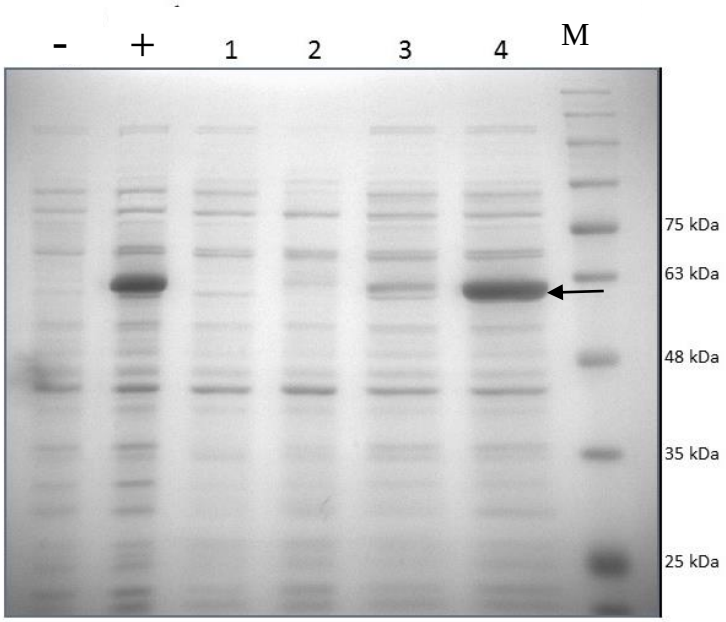

Fig. 4.11. Effect of incubation temperature and time on TrxHisS-CBFIIId-D17 protein extraction from *E.coli*. SDS-PAGE analyses with Coomassie blue stain of total cellular proteins before (─) and after (+) induction with IPTG and (1) total soluble protein extracted by incubating the cells at 38°C water-bath with 120 rpm shaker for 10 min, (2) similar conditions as (1) but incubated for 20 min, (3), total soluble proteins extracted by passing the cell suspension through Emulsiflex C3 homogenizer for 2 min, (4) same as (3) but passed through the homogenizer for 5 min, M; Protein molecular weight marker. Migration of the CBF is marked by an arrow on the gel (→).

After SDS-PAGE analyses and staining with Coomassie brilliant blue, the 12% polyacrylamide gel revealed the presence of a polypeptide corresponding to TrxHisS-CBFIIId-D17 in the induced cells and total cell extract (Fig. 4.11). Homogenizing the cells at 15,000 psi for 2 min increased the amount of TrxHisS-CBFIIId-D17 in the soluble fraction, but the improvement was little (lane# 3). However, the amount of TrxHisS-CBFIIId-D17 (lane# 4, Fig. 4.11) was significantly increased with 5 min of exposure to high pressure during protein extraction. The method optimized for TrxHisS-CBFIIId-D17 was also used for TrxHisS-CBFIVb-A20 (putative), -CBFIVb-21.1 (putative) and CBFIVd-A22. SDS-PAGE analyses of total cellular proteins, before and after induction and in cell soluble fraction show the presence of corresponding TrxHisS-CBF polypeptides (data not shown). In conclusion using NPI-10 protein extraction buffer to re-suspend the cells and then disrupting the cells under high pressure by a homogenizer can be used to obtain several recombinant CBFs in cell soluble extracts (Table 4.2).

4.3.5. Anomalies in TrxHisS-CBF migration during SDS-PAGE analysis

SDS-polyacrylamide gel electrophoresis analyses revealed that the majority of the TrxHisS-CBFs migrated significantly slower than expected based on their predicted molecular weights. Thus the apparent molecular weight was higher than their predicted molecular weight for all the recombinant CBFs studied except those in subgroup IVd (Table 4.3). The highest deviation could be observed in the CBFs from subgroup IIId (TrxHisS-CBF12 and -CBF15 isoforms, -CBFIIId-D17, -CBFIIId-D19), there was an average of 25% deviation from their calculated molecular weight. The CBFs from subgroup IVa (TrxHisS-CBF2 isoforms) and IVb (TrxHisS-CBF20 and -CBF21 isoforms) showed an average deviation of 15-20%. The only exception was CBF members from subgroup IVd (TrxHisS-CBF9 and -CBF22 isoforms), which showed either no variation (TrxHisS-CBFIVd-B9 and -B5-CBF22) or migrated faster (TrxHisS-CBFIVd-D9 and CBFIVd-B22) than expected (Table 4.3).

4.4. Purification of recombinant TrxHisS-CBF fusion proteins

Recombinant CBF fusion proteins were purified using affinity chromatography and ion exchange chromatography procedures. For affinity chromatography one mL HisTrapFF affinity chromatography columns and for ion exchange chromatography one mL HiTrapQXL ion exchange columns (GE Healthcare, Mississauga, ON, Canada) were used. SDS-PAGE analyses

Table 4.2. Efficiency of the three protein extraction methods used in this study

CBF subgroup	CBF* designation	Genome location	Method #		
			1.Bugbuster	2.NPI-10	3.Physical
IIId	IIId-B12	5A/5B	+	+	++
	IIId-A15	5A/5B	+	+	++
	IIId-D17**	5A	-	-	++
	IIId-D19	5B	-	-	++
IVa	IVa-A2 like	5D	-	+	++
	IVa-A2	5D	+	+	++
	IVa-2.3	5D	-	-	++
IVb	IVb-B20	5B/5D	-	-	++
	IVb-A20**	5A	-	-	++
	IVb-D21	5D	-	-	++
	IVb-21.1**	5A	-	-	++
IVd	IVd-B9	5B	+	+	++
	IVd-D9	5D	-	+	+
	IVd-A22**	5D	-	-	+
	IVd-B22	5A/5B	-	-	+

* CBF designation based on Badawi *et al*., 2007 and MacLachlan *et al*., 2011

** Cloned in a previous study (Jain, 2013)

– Less efficient; TrxHisS-CBFs were degraded (yield could not be determined)

+ Efficient; some of the TrxHisS-CBFs could be obtained in intact forms (0.1-0.7 pmol target protein yield)

++ Most efficient; All of the TrxHisS-CBFs could be obtained in intact forms (0.5-4 pmol target protein yield)

Table 4.3. Gel migration characteristics of the TrxHisS-CBFs studied

Trx-HisS-CBF[¶]	BAC clones[*]	Molecular mass (kDa)				
		Predicted	Trx tag	Calculated total	Observed	% difference
IIId-B12	1558M10	25.8	17.08	42.88	56.0	30
IIId-A15	1558M10	25.5	17.08	42.58	51.0	21
IIId-D17[**]	425P7	30.4	17.08	47.48	60.0	25
IIId-D19	1179D19	24.8	17.08	41.88	52.0	23
IVa-A2	260E13	21.6	17.08	38.68	50.0	28
IVa-A2 like	926I25	24.5	17.08	41.58	50.0	19
IVa-2.3	926I26	25.4	17.08	42.48	49.0	14
IVb-B20	1374F6	23.3	17.08	40.38	47.0	17
IVb-A20[P**]	567H13	23.9	17.08	40.98	46.0	12
IVb-D21	1374F6	22.2	17.08	39.28	46.0	17
IVb-21.1[P**]	567H13	21.6	17.08	38.68	45.0	15
IVd-B9	653A22	29.2	17.08	46.28	45.0	-3
IVd-D9	1548G4	28.7	17.08	45.78	46.0	~0
IVd-A22[**]	408B5	29.8	17.08	46.88	46.0	~0
IVd-B22	341J20	32.0	17.08	49.08	45.0	-8

[¶] CBF designation based on Badawi *et al*., 2007 and MacLachlan *et al*., 2011 ([P]; Putative CBF proteins)

* Clone number in Norstar BAC library (Ratnayaka *et al*., 2005)

[**] Cloned in a previous study (Jain, 2013)

of total cellular extracts before and after induction as well as the cell soluble fractions and steps during column chromatography revealed the enrichment of a protein band corresponding to TrxHisS-CBFIIId-A15 in the induced cells and the cell soluble fraction. Corresponding bands were either missing or had very low intensity in the flow through, wash buffer or binding buffer fractions. The TrxHisS-CBFIIId-A15 band was visible in high intensity in the eluted fraction. However, a few other minor contaminating bands were visible (Fig. 4.12). This suggested that the purification was not complete and there were losses during the purification steps. To remove minor contaminants, ion exchange chromatography using HiTrapQXL (GE Healthcare, Mississauga, ON, Canada) was used to purify the eluted fractions after affinity chromatography. However, due to increased loss of recombinant proteins this step was not pursued further.

4.4.1. Fast liquid chromatography (FPLC) based automated purification

An automated fast protein liquid chromatography (FPLC) system (AktaPure, GE Healthcare) was used to improve the efficiency of the TrxHisS-CBF purification. In this system protein purification was controlled by the software supplied by the company (Unicorn 6.3; Fig. 4.12A). The starting sample for protein purification could be as high as 150 mL but for this study, the volume was restricted to only 40-50 mL. Proteins of interest (TrxHisS-CBFs) were eluted using a gradient of imidiazole in the elution buffer. SDS PAGE analyses of TrxHisS-CBFIIId-B12 total cellular proteins before and after induction showed the presence of a strong polypeptide band corresponding to TrxHisS-CBFIIId-B12 in the induced cell extract (Fig. 4.12). Similar corresponding polypeptide was observed in the eluted fractions. The earlier fractions eluted with low imidazole conc. contained most of the contaminating bands, but the later fractions with higher imidazole conc. were relatively pure (Fig. 4.12). Most of the TrxHisS-CBFs could be eluted at an imidazole conc. of 150-200 mM (Fig. 4.12A). This showed that the gradient elution removed most of the contaminating proteins at lower concentrations of imidazole in the elution buffer. The eluted fractions were collected by a fraction collector. Only the fusion protein fractions with the highest purity could easily be selected for further studies. This protocol was used to purify all the TrxHisS-CBFs studied in this project (Table 4.3; Fig. 4.13).

4.4.2. Desalting and storage of the purified TrxHisS-CBF proteins

Two procedures were followed to remove excess sodium chloride and imidazole from the

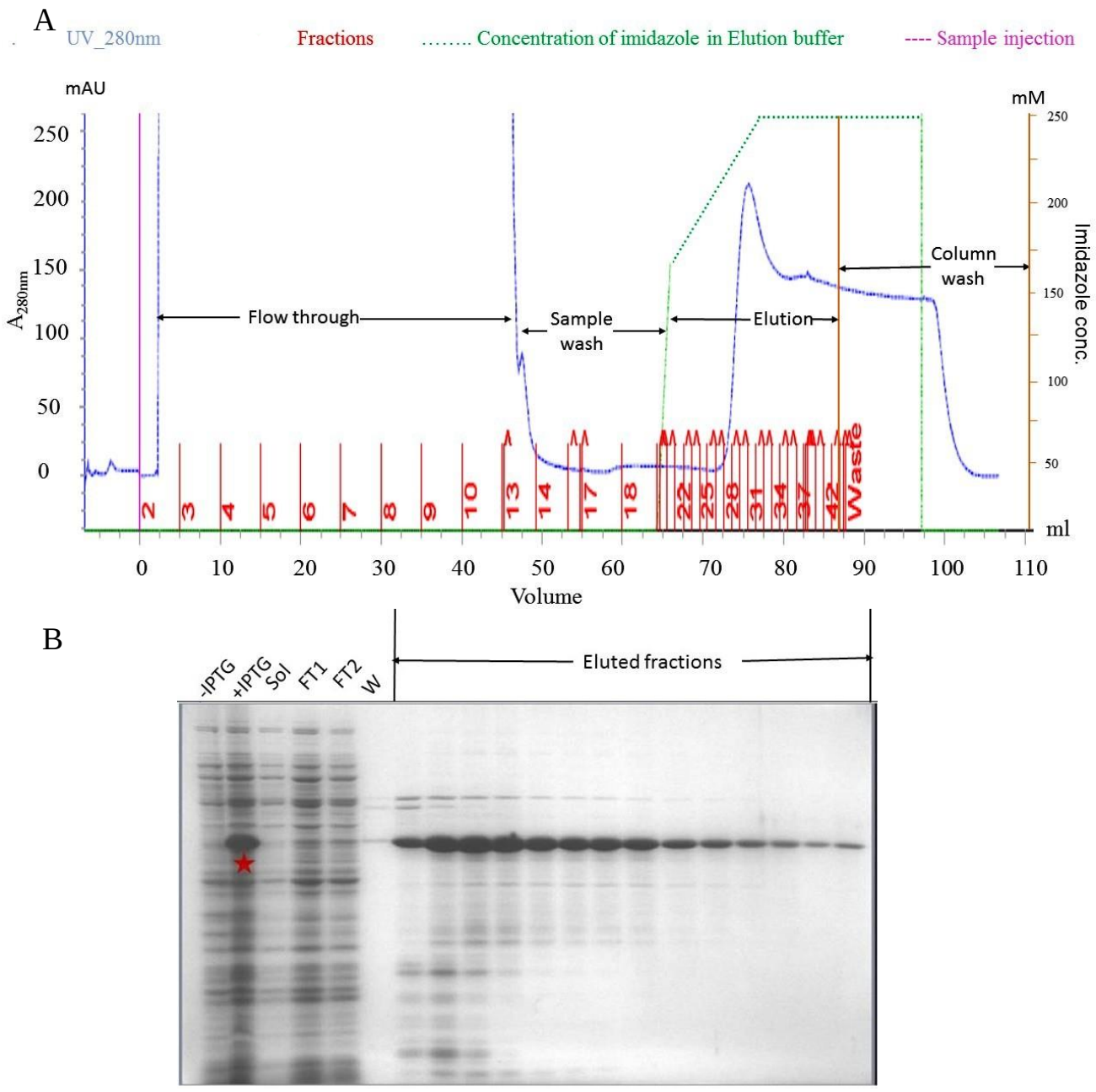

Fig. 4.12. Purification of TrxHisS-CBFIIId-B12 from *E. coli* soluble extract using AKTAPure™ FPLC system (GE Healthcare). A; Sensogram generated by the Unicorn 6.3 software during automated purification. B; SDS-PAGE analysis of fractions generated during purification. Total cellular proteins before (─), and after (+) complete induction, cell soluble (Sol), flow through (FT1), flow through 2 (FT2), wash (W) and eluted fractions with increasing imidazole concentration. Each flow through fraction contained 5 mL sample for the total volume of extracted fraction, whereas, purified samples were collected as 1 mL fractions.

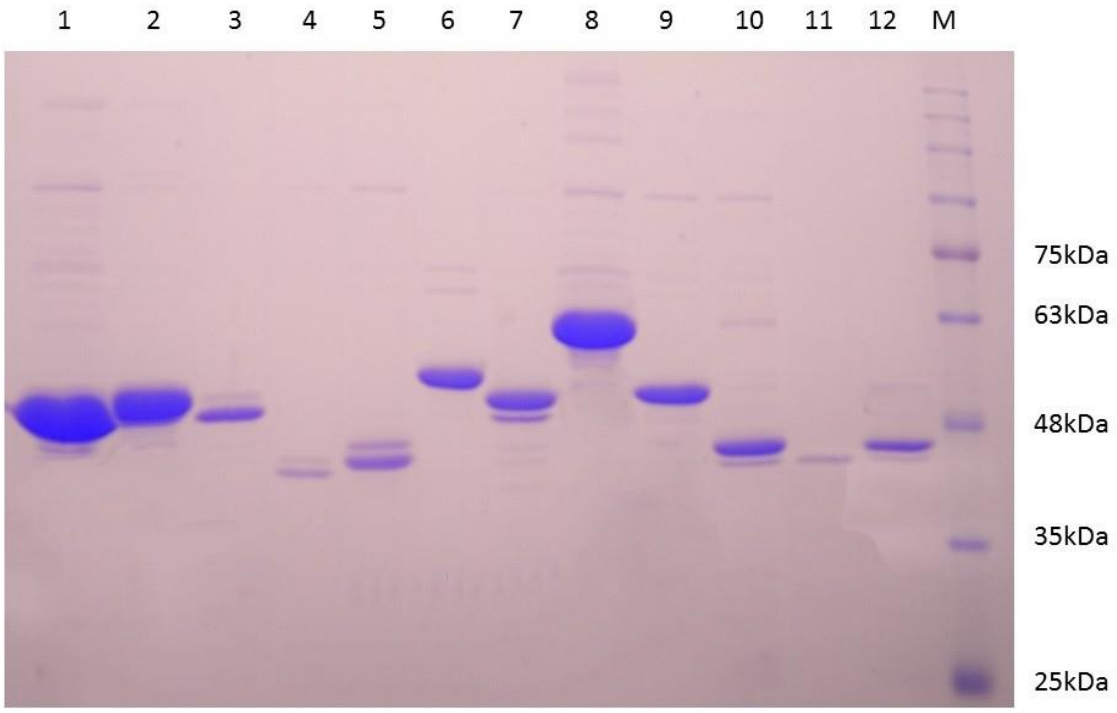

Fig. 4.13. Recombinant TrxHisS-CBF fusion proteins purified in this study. SDS-PAGE analysis of the purified recombinant CBFs on a 12% polyacrylamide gel stained with Coomassie blue: Lanes# 1, CBFIVa-A2; 2, CBFIVa-A2 like; 3, CBFIVa-2.3; 4, CBFIVd-D9; 5, CBFIVd-B9; 6, CBFIIId-B12; 7, CBFIIId-A15; 8, CBFIIId-D17; 9, CBFIIId-D19; 10, CBFIVb-B20; 11, CBFIVb-A20 (putative); 12, CBFIVb-D21.

eluted CBF fusion protein fractions. In one protocol, purified TrxHisS-CBF proteins were dialyzed against 2 L of a low salt containing buffer (20 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0) overnight with three buffer changes in between. In the other protocol, eluted recombinant CBFs were passed through a desalting column (HiTrapFF, GE Healthcare, Mississauga, ON, Canada) and then, re-eluted against 5 mL of the same buffer mentioned above. After elution, glycerol was added to the proteins to a final concentration of 10% (v/v). Samples were flash frozen in liquid nitrogen and stored at -80°C until further use. Some of the low molecular mass contaminating proteins could be removed in this process because of the molecular cut off size of dialysis tubes.

4.5. Quantification of the TrxHisS-CBFs

Following the dye binding assay (Bradford, 1976), the concentration of proteins in the purified fractions were calculated to vary from 0.32 to 1.44 mg mL^{-1}. TrxHisS-CBF2 isoforms had the highest concentration of purified protein. The N-terminal tag in the recombinant proteins contained an S-tag (15 amino acid long pancreatic RNAase A; Kim & Raines, 1993). To calculate the exact amount of target recombinant protein an S-tag based assay kit was used, which is based on the interaction between this S-tag oligopeptide and ribonuclease S protein (described in section 3.12; Richards & Wyckoff, 1971). The S-tag assay kit revealed the presence of 26-72% target recombinant TrxHisS-CBF proteins in purified fractions (Table 4.4).

4.6. Bioinformatics analyses of the CBFs studied

The secondary protein structures are made of α-helices, β-sheets and β-turns. These structures help in the formation of stable, active three dimensional structures of the proteins. The AP2 domain region of the CBFs contains three β-sheets and one α-helix (Table 4.5, 4.7; Fig. 4.14). In addition, a few β-turns could also be observed in the CBF coding region. Subtle unique amino acid changes could be observed in the AP2 domain of the CBFs that differ in their binding abilities to the *Cor* promoters (Table 4.6). It was also observed that their AP2 binding domain in the non-binder CBF had deletion of 4-5 amino acids compared to the DNA binding CBFs (Table 4.6). From this study, the lowest percentage of β-turns could be predicted in the CBFs belonging to the subgroup IIId, whereas the highest percentage of β-turns could be observed in subgroup IVb (Table 4.7).

Table 4.4. Quantification of purified TrxHisS-CBF proteins

Trx-HisS-CBF designation[¶]	Quantification of purified recombinant CBFs				
	Concentration (mg mL^{-1})*	Vol. (mL)	Total Yield (mg)	Concentration (pmol) **	% of total protein ***
CBFIIId-B12	0.6	5	3.0	1.7	47
CBFIIId-A15	0.38	5	1.9	0.5	41
CBFIIId-D17	1.08	5	5.4	5.5	61
CBFIIId-D19	0.47	5	2.3	3.9	59
CBFIVa-A2	1.11	5	5.6	4.2	72
CBFIVa-A2like	1.44	5	7.2	3.8	57
CBFIVa-2.3	0.71	5	3.7	4.2	28
CBFIVb-D9	0.38	5	1.9	3.8	51
CBFIVb-B20	0.44	5	2.2	1.7	32
CBFIVb-D21	0.32	5	1.6	1.1	26

[¶] CBF designation based on Badawi *et al*., 2007

* Concentration was measured by using a dye binding assay (Bradford's assay)

** pmol concentration was determined by using S-Tag assay system kit (Novagen)

*** Percentage calculation was performed following the protocol described in the S-Tag assay kit

[¥] Recombinant CBF proteins obtained from 250 mL *E. coli* cultures

Table 4.5. Comparative analysis of AP2 domain with the flanking conserved amino acids from CBF 2 isoforms*

CBFs	Sequence of the AP2 domain with the flanking conserved motifs	
IVa-A2	**PKRRAGR**IKLQETRHPVYR*GVRRR*GRVGQ*WVCELR*VPASRGYS	43
IVa-A2 like	**PKPRAGR**NKLQETRHPVYR*GVRRR*GREGQ*WVCELR*VPAA-GSR	43
IVa-2.3	**PKRRAGR**NKLQETRHPVYR*GVRRR*GREGQ*WVCELR*VPAGSRSY	43
	β1 β2	
IVa-A2	-----*RLWLG*TFATAEMAARAHDSAALALSGHDACLNFA**DSAWR**	85
IVa-A2 like	VYS*RIWLG* TFADPEMAARAHDSAALALSGRDACLNFA**DSAWR**	85
IVa-2.3	S---*RIWLG* TFASAQMAARAHDSAALAL SGRDACLNFA**DSAWR**	85
	β3 α-helix	

* Alignment of the CBF2 isoforms and their AP2 domain with the flanking conserved amino acid sequences (in bold letters). Sequences of the β-sheets are italicized and the α-helix is in grey color and underlined (AGADIR, APSSP; Yi & Lander, 1993). This alignment was done by online available tool COBALT (www.ncbi.nlm.nih.gv; Dereeper *et al.*, 2010).

Table 4.6. Sequence homology comparison of the AP2 DNA binding domains of the DNA binder and non-binder CBFs studied*

CBF	Sequence alignment of the AP2 domain with the flanking conserved motifs	
IIId-B12	**PKRPAGR**TKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGER	43
IIId-D19	**AKRPAGR**TKFKETRHPVYRGVRRRGSAGRWVCEVRVPGKRGER	43
IVa-A2	**PKRRAGR**IKLQETRHPVYRGVRRRGRVGQWVCELRVPASRGYS	43
IVa-A2 like	**PKPRAGR**NKLQETRHPVYRGVRRRGREGQWVCELRVPAAGSRV	43
IVa-2.3	**PKRRAGR**NKLQETRHPVYRGVRRRGREGQWVCELRVPAGSRSY	43
IIId-A15	**LKRPAGR**TKFKETRHPVYRGVRRRGS<u>A</u>GRWVCEVRVPGKR<u>E</u>RL	43
IVd-B9	**PKRPAGR**TKFHETRHP<u>L</u>YRGVRRRGRVGQWVCEVRVPG<u>I</u>K<u>G</u>SRL	43
IIId-B12	LWLGTHVTAEAAARAHDAAMLALYGRTP----AARLNYP**DSAWL**	85
IIId-D19	LWLGTYAAAESAARAHDAAMLALL-GHSASAAACLNFP**DSAWL**	85
IVa-A2	---RLWLGTFATAEMAARAHDSAALALSGH----DACLNFA**DSAWR**	85
IVa-A2 like	YSRIWLGTFADPEMAARAHDSAALALSGR----DACLNFA**DSAWR**	85
IVa-2.3	--SRIWLGTFASAQMAARAHDSAALALSGR----DACLNFA**DSAWR**	85
IIId-A15	------WLGTHLTAEAAARA<u>Y</u>DAAMLCLIGPS<u>TQ</u>----CLNFA**DSAWL**	85
IVd-B9	------WLGTFNTAEMAARAHDAAVLALSGRA----ACLNFA**DSAWR**	85

*Alignment of the AP2 binding domain and the flanking conserved amino acid residues (in bold letter) of the DNA-binding and non-binding CBFs (Dereeper *et al.,* 2010)). Unique amino acid changes in the non-binding CBFs are underlined. DNA non-binding CBFs are within the box.

Table 4.7. Bioinformatics analyses of CBFs studied*

CBF**	Secondary structural prediction for CBFs (%)				PEST score	Net charge	Instability index
	α-Helix	β-Sheets	β-Turns	Aromatic amino acids			
CBFIIId-B12	53	38	13	5.7	+9.12	+7	57.58
CBFIIId-A15	58	49	13	7.5	-0.01	+10	64.62
CBFIIId-D19	69	53	10	9.5	+6.15	+6	56.82
CBFIVa-A2	69	50	12	10.0	-0.74	+4	53.52
CBFIVa-A2like	63	40	15	10.3	+2.81	+2	47.32
CBFIVa-2.3	68	44	16	10.2	+10.69	+4	56.04
CBFIVb-B9	71	45	13	6.0	-11.11	+4	66.04
CBFIVb-D9	77	44	13	6.6	-12.15	+7	68.05
CBFIVb-B20	51	49	15	8.5	+12.53	+10	47.24
CBFIVb-D21	60	42	16	8.4	+11.36	+9	49.72

* Bioinformatics studies were performed using the ExPASy proteomics tools (http://www.expasy.org).

** CBF designations based on Badawi *et al*., 2007.

The presence of PEST sequence (proline, glutamic acid, serine and threonine) is an indicative of protein instability (Rechsteiner & Rogers, 1996). Based on the PEST score prediction, CBFs from subgroup IVd appeared to be the most stable CBFs (Table 4.7). Net charge and amino acid constituents affect the migration of proteins during polyacrylamide gel electrophoresis and hence, studying these properties could help to explain reasons for slow migration during PAGE analyses of recombinant CBFs. This study revealed that the net charge of the CBFs ranged from +4 to +10, while their aromatic amino acid constituents varied from 5-10% (Table 4.7). A phylogenetic tree was generated (Fig. 4.14; www.phylogeny.fr; Dereeper *et al.*, 2008) on the basis of the AP2 domain and the CMIII-1 and CMIII-3 flanking conserved amino acid residues. High support value of 0.99 for the two basic nodes in this tree suggests that these two branches are close together from evolutionary point of view (Fig. 4.14). Sequence-wise, two CBF9 isoforms were found to be distantly related, whereas the three CBF2 isoforms showed a higher support value of 0.98, suggesting they are evolutionary related (Fig. 4.14).

4.7. Observation of DNA/protein interaction by electrophoretic mobility shift assay

4.7.1. TrxHisS-CBFs have varied affinity towards CRT motif *in vitro*

Binding equal concentrations of purified TrxHisS-CBFs to equimolar concentrations of end labeled *Wcs120* promoter DNA carrying the CRT motif for 20 min showed varied level of binding to the CRT motifs (Fig. 4.15; Table 5.1). The CBFs from group IVa bound strongly to the CRT motif than CBFs from any other group. Three TrxHisS-CBF2 isoforms from this group were tested for their DNA binding ability. The TrxHisS-CBFIVa-A2 showed the highest level of binding, followed by TrxHisS-CBFIVa-A2 like. The TrxHisS-CBFIVa-2.3 showed poor binding to the CRT motifs. Subgroup IVa was followed by subgroup IIId in terms of DNA binding ability where, TrxHisS-CBFIIId-B12 had the best binding, followed by TrxHisS-CBFIIId-D17 and TrxHisS-CBFIIId-D19 respectively. TrxHisS-CBFIIId-A15 did not bind nor did TrxHisS-G4-CBF 9 from subgroup IVd. Limited data obtained in this study the CBFs binding ability could be arranged in the following sequence:

TrxHisS-CBFIVa-A2> -CBFIVa-A2 like > -CBFIIId-B12> -CBFIIId-D17> CBFIIId-D19> -CBFIVa-2.3> - CBFIIId-A15, -CBFIVd-B9

4.7.2. Effect of temperature on *in vitro* DNA binding ability of TrxHisS-CBFs

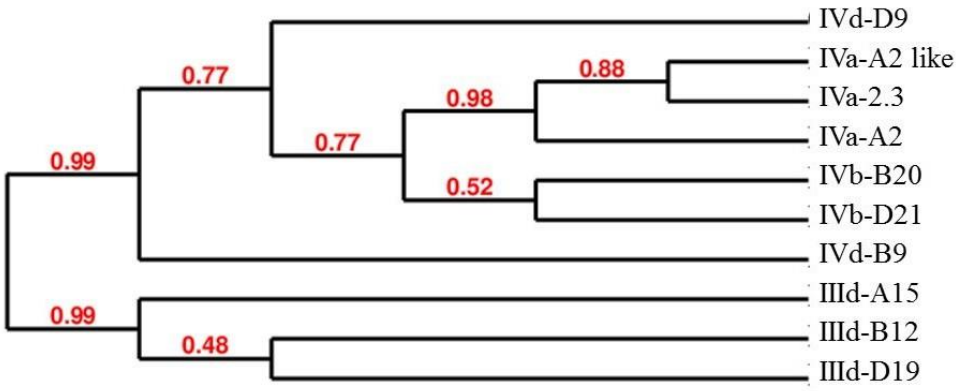

Fig. 4.14. Phylogenetic tree generated by comparing the AP2 DNA binding domain and the flanking sequences of the CBFs studied. This tree was generated using an online tool available at www.phylogeny.fr (Dereeper et al., 2008). High support value of the two basic nodes in this tree suggests that these two branches are similar from evolutionary view point (support value 0.99). On the other hand CBFIIId-A15 was distant from the other two members of the same clade (support value 0.48).

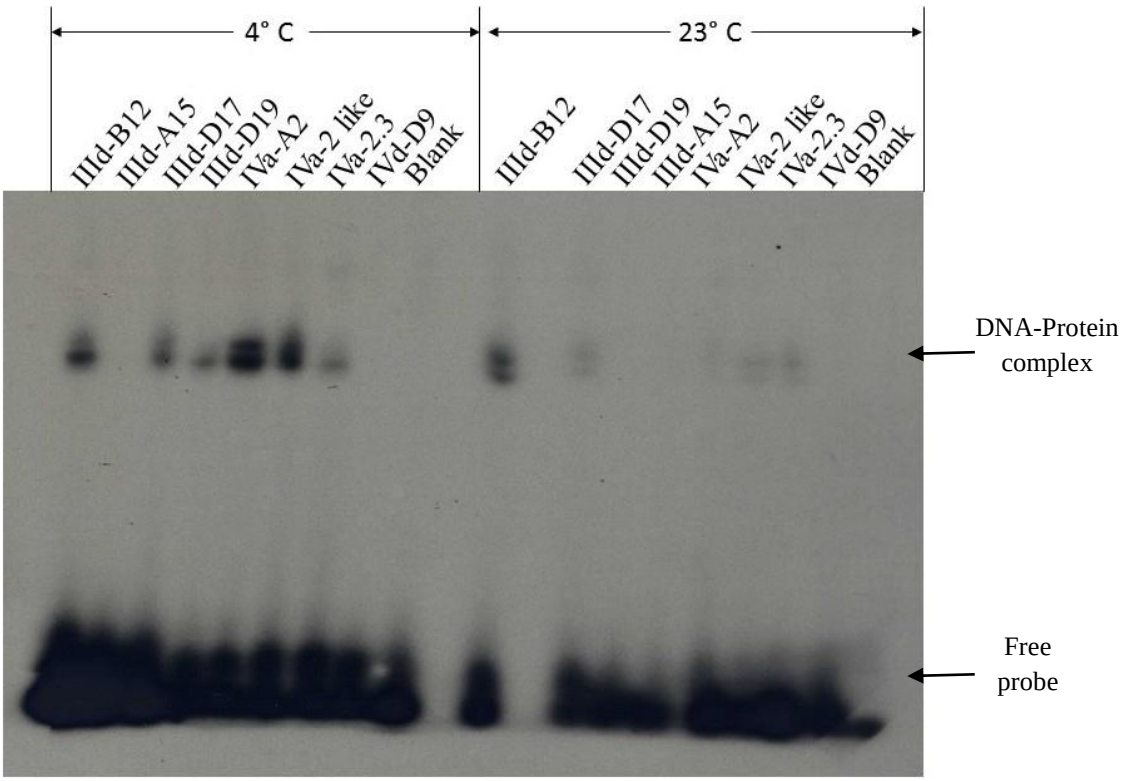

Fig. 4.15. Effect of incubation temperature on DNA binding intensity of the TrxHisS-CBFs assessed in the EMSA experiment. Increased binding intensity for most of the recombinant CBFs was observed when incubated at 4°C, than at 23°C. TrxHisS-CBFIIId-A15 failed to show any DNA binding at any of these two temperatures.

To test the effects of temperature on the DNA binding ability of TrxHisS-CBFs, the incubation was performed at room temperature (23°C) as well as, at 4°C for 20 min. The gel radiograph clearly showed increased binding of TrxHisS-CBFs to CRT motif at 4°C temperature (Fig. 4.15). Surprisingly, TrxHisS-CBFIIId-D19 could only bind to the CRT motif when incubated at 4°C *in vitro*. On the contrary, TrxHisS-CBFIIId-B12 could bind to the CRT motif with almost equal intensity, irrespective of the incubation temperature (Fig, 4.15).

4.7.3. Effect of incubation time on *in vitro* DNA binding ability of TrxHisS-CBFs

To test the effect of incubation time on the DNA binding ability of these recombinant CBF proteins *in vitro*, similar binding assays were performed except that the incubation time was increased to 60 min instead of 20 min Under this condition TrxHisS-CBFIIId-B12 showed improved binding as did TrxHisS-CBFIIId-D17. On the other hand, two of the three TrxHisS-CBF2 isoforms did not show any significant increase in their binding pattern except TrxHisS-CBFIVa-2.3, which increased considerably. Presence of a very faint band in the case of TrxHisS-CBFIVd-B9 could be due to very little binding. TrxHisS-CBFIIId-A15 could not show any binding to the CRT region. Surprisingly, the dual bands observed in case of the better binding CBFs when incubated at 4°C for 20 min disappeared when incubated for 60 min. Results also showed that at reduced incubation time, both TrxHisS-CBFIIId-B12 and -CBFIIId-D17 showed poor binding compared to TrxHisS-CBFIVa-A2 but, when incubated for a prolonged time, TrxHisS-CBFIIId-B12 was the best binder among all the TrxHisS-CBFs tested. Limited results at two binding temperatures obtained in this study suggest the following pattern of the TrxHisS-CBFs *in vitro* affinity towards CRT motif:

TrxHisS-CBFIIId-B12> -CBFIIId-D17> -CBFIVa-A2> -CBFIVa-A2 like > -CBFIIId-D19> -CBFIVa-2.3> -CBFIVd-B9> -CBFIIId-A15

4.8. Surface plasmon resonance (SPR) analyses for DNA/protein interaction

4.8.1. Binding of TrxHisS-CBFs to CRT motif

Varying concentrations (20 nM to 1.25 nM) of biotinylated COR DNA fragments were immobilized on different channels of the chip surface to determine the optimal concentration of DNA required for the DNA-protein interaction. One mM concentrations of four TrxHisS-CBFs

(TrxHisS-CBFIIId-B12, -CBFIIId-A12, -CBFIIId-A15, -CBFIIId-B15) were flown over biotinylated COR DNA fragments. The sensogram showed that three of the four TrxHisS-CBFs could bind to COR DNA fragment and that they varied in their binding intensity (Fig. 4.16). No curvature was observed with TrxHisS-CBFIIId-A15 that could indicate a positive interaction. Previously it was thought that perhaps the graphical anomaly resulted due to stickiness. But, latter this result partly supported the non-binding of TrxHisS-CBFIIId-A15 in EMSA experiment (Fig. 4.15, 4.16). To calculate the binding kinetics, maximum concentration of ligand required to obtain a 100 response units (RU) was calculated to be 20 nM, where 1 RU corresponds to 1 ng mm^{-2} binding.

4.8.2. Real time comparison of CRT binding properties between two TrxHisS-CBF12 isoforms

Two isoforms of TrxHisS-CBF12 used in this experiment are encoded by *FrA2* (CBFIIId-A12) and *FrD2* (CBFIIId-B12) are 72% identical at amino acid level as observed from BLAST search (data not shown). Five different concentrations ranging from 1 μM to 12.5 nM at one third serial dilutions were flown over 20 nM of biotinylated COR DNA fragments immobilized on the chip surface (Fig. 4.17). The sensogram showed variations in the binding ability of the two TrxHisS-CBF12 isoforms. The association rate (K_a), dissociation rate (K_d), the affinity of the protein-DNA complex (K_D) suggested that association rate towards the COR DNA CRT motif for TrxHisS-CBFIIId-B12 ($9.64E^{+04}$ 1/Ms) is almost nine times higher than that of TrxHisS-CBFIIId-A12 ($1.11E^{+04}$ 1/Ms). In contrast TrxHisS-CBFIIId-A12 has higher K_d value ($3.47E^{-02}$ 1/s) than TrxHisS-CBFIIId-B12 ($2.60E^{-02}$ 1/s). This was reflected from the affinity of the interaction (Fig. 4.17C, D). Because, TrxHisS-CBFIIId-B12 had a higher association rate but a lower dissociation rate, the former showed better affinity towards the CRT motif. It is interesting to note that both the isoforms of TrxHisS-CBF12 examined in this experiment were quite similar at amino acid level, but functional differences exist between them. In the case of two isoforms of TrxHisS-CBF15, TrxHisS-CBFIIId-A15 did not bind, hence no comparison was possible except that TrxHisS-CBFIIId-B15 had a low level of DNA binding affinity towards the CRT motif, compared to TrxHisS-CBF12 isoforms.

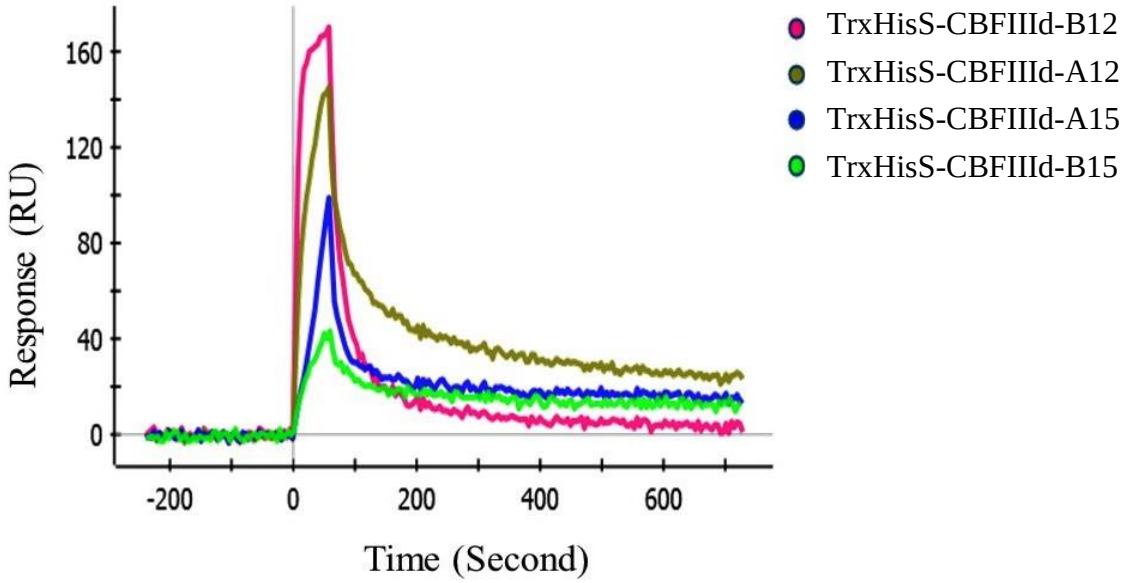

Fig. 4.16. Sensogram obtained from the interaction between TrxHisS-CBFs and the biotinylated *Wcs120* promoter fragment encoding the CRT motif. TrxHisS-CBFIIId-B12 (*FrD2*) had the highest binding (pink peak on the graph) with its higher RU value. This was followed by TrxHisS-CBFIIId-A12 (*FrA2*; dark green peak in the graph). Both the TrxHisS-CBF15 isoforms showed weak binding than the TrxHisS-CBF12 isoforms. TrxHisS-CBFIIId-B15 bound the least (*FrD2;* green peak in the graph). TrxHisS-CBFIIId-A15 (*FrA2)* did not show any binding at all, which was evident from its unusual peak (blue). One RU corresponds to 1 ng mm^{-2} binding affinity.

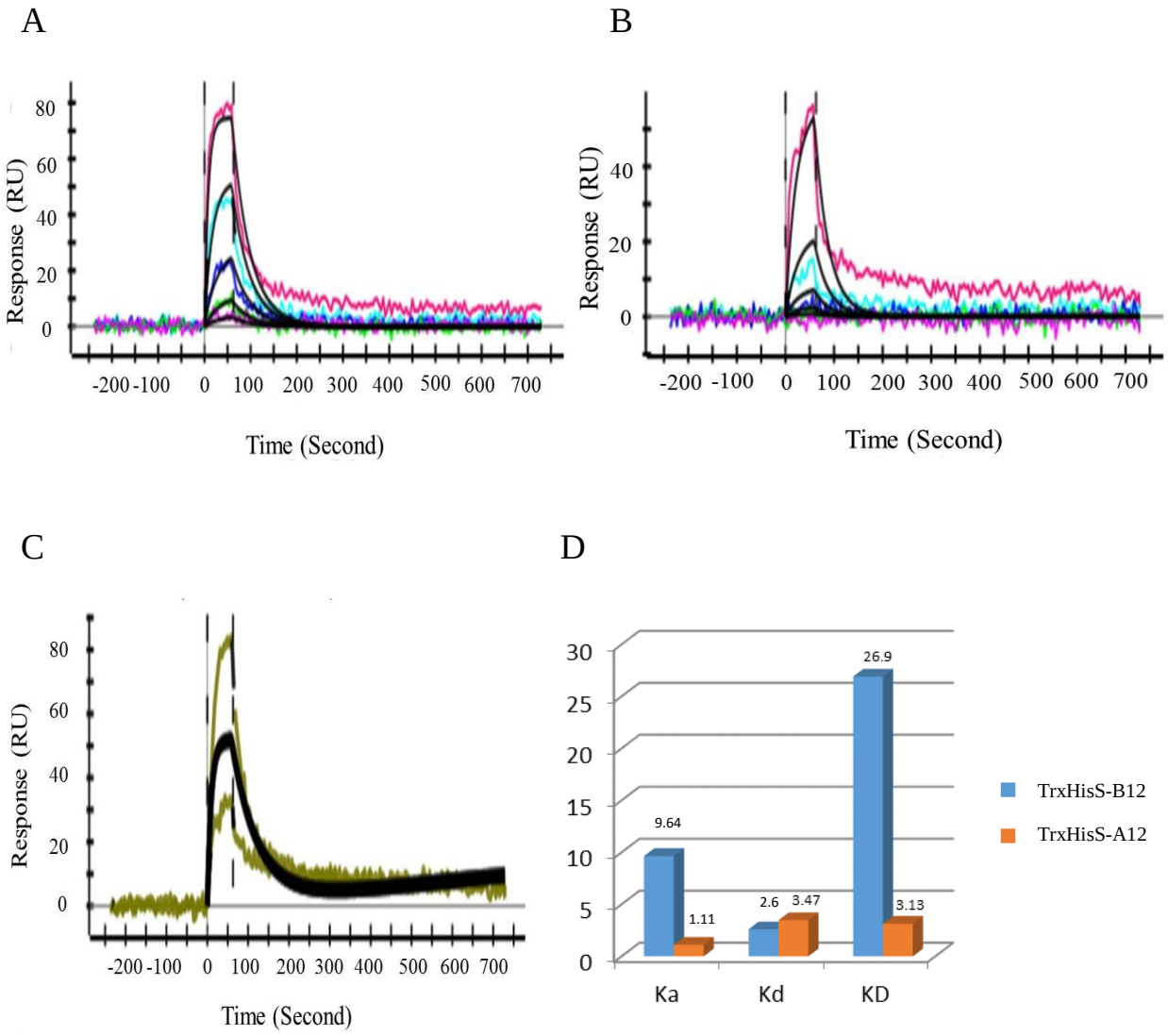

Fig. 4.17. Sensograms obtained from the interaction of various concentrations of two TrxHisS-CBF12 isoforms with 20 nM biotinylated COR DNA fragment. A; CBFIIId-B12 and B; CBFIIId-A12. In both the cases, best binding reaction occurred with 1 µM protein concentration (red peak in the figures). C; Sensogram obtained from the comparison of DNA binding ability of the TrxHisS-CBF12 isoforms. D; A bar graph representing the values obtained in sensogram C. K_a; Association constant, K_d; Dissociation constant, K_D; Affinity of the reaction. One RU corresponds to 1 ng mm^{-2} binding reaction.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1. Mechanical disruption is necessary for *E. coli* protein solubilization

Cloning and expression of recombinant proteins in *E. coli* have been in the practice for more than three decades. The prokaryotic origin of CBFs (Magnani *et al*., 2004), has enabled us to use a bacterial protein expression system appropriately. The T7 promoter based pET system in relation to over-expression of fusion proteins was first mentioned almost 25 years ago (Dubendorff & Studier, 1991) and has since been commercialized by Novagen and successfully used for various expression studies (Sørensen & Mortensen, 2005b). For protein functional studies it is important to obtain the recombinant protein in its native non-denatured form and to its highest purity. To produce recombinant proteins, *E. coli* strain BLR(DE3)pLysS is suitable as it has mutations to increase stability of plasmid monomers and the production of fusion proteins is under the tight control of *LacUV5* promoter that can be induced by metabolically inactive IPTG. These cells also have little to no protease activity, which is important for a foreign protein to grow inside the *E. coli* cells.

In this study, 12 CBF genes cloned in *E. coli* were successfully over-expressed as TrxHisS-fusion proteins. Out of all the combinations tested, the optimal method was to grow the cells at 28°C until the culture reached an A_{600nm} value of 0.3. At this stage, to induce recombinant protein production IPTG was added to the culture and grown for next 3 h or overnight (TrxHisS-CBFIVd-B22) to obtain an adequate amount of fusion protein. For recombinant protein extraction, several buffer compositions were used. NPI-10 buffer was found to be the most suitable buffer to obtain the proteins in cell soluble extracts. Unfortunately, some of the recombinant CBFs, namely: TrxHisS-CBFIIId-D19, -CBFIVb-B20, -CBFIVb-D21, -CBFIVd-B22 could not be obtained in the cell soluble extracts using only this buffer. In a previous study, some isoforms of TrxHisS-CBF17, -CBF20, -CBF21 and -CBF22 formed aggregates and could only be extracted in denatured condition (Jain, 2013).

The three layers of cellular envelopes of *E. coli* (Hammond *et al*., 1984; Harrison, 1991) must be disrupted to obtain the cellular proteins in the cell soluble extracts. Incubation in buffer or undergoing freeze-thaw cycle(s) probably was not adequate to disrupt these many layers of

protective envelope. In a previous study, mechanical pressure using a high pressure homogenizer was necessary to disrupt microbial cells (Engler & Robinson, 1981). A combination of freeze-thaw cycle and mechanical pressure using a cell homogenizer was used to disrupt the bacterial cells in this study and was shown to be the most suitable method to obtain cellular proteins in the cell soluble extract.

The use of a mechanical disruption procedure by a cell homogenizer not only facilitated disruption of the cellular protective layers, but also was an optimal method to obtain aggregating CBFs in cell soluble extracts. It is quite likely that high pressure could break the protein aggregates and improved their solubility. The cell disruption method optimized in this study was successfully used to extract the four CBF isoforms (TrxHisS-CBFIIId-D17, -CBFIVb-A20, -CBFIVb-21.1 and -CBFIVd-A22) in cell soluble extract, which was not possible otherwise (Jain 2013).

5.2. Automated FPLC based protein purification is more efficient than manual purification

For both manual and automated purification procedures, nickel affinity chromatography columns obtained from GE Healthcare were used. Manual purification was time consuming, yields were low, nor could the starting material be increased in volume. The automated purification procedure using the AktaPure™ FPLC system (GE Healthcare) significantly improved the purification quality and capacity. A starting material of more than 50 mL could be used for purification, thus more recombinant protein could bind to the nickel resin and as a result, an increased amount of protein could be eluted as purified fractions. Also, a linear gradient of imidazole in the elution buffer used during FPLC purification method, removed most of the contaminating proteins during the early stages of purification. Thus the highest purity of recombinant TrxHisS-CBFs was obtained using the automated FPLC method. These TrxHisS-CBF fusion proteins withstood repeated freeze and thaw cycle thus exhibiting stability over a range of temperatures, as reported in a previous study (Jain, 2013).

5.3. Structural moiety of some TrxHisS-CBFs might be responsible for slow migration during SDS-PAGE

When subjected to electrophoresis on 12% SDS-polyacrylamide gels, most of the TrxHisS-CBFs migrated slower than expected. Thus, their apparent size on the gel appeared to

be higher than their calculated molecular mass. The highest deviation was observed for TrxHisS-CBFs from subgroup IIId where on average a deviation of 20-30% could be seen in between expected and observed size. Only exceptions were TrxHisS-CBF9 and -CBF22 isoforms from subgroup IVd. These CBFs migrated normally compared to their theoretical molecular weight and were close to their expected molecular mass. TrxHisS-CBFs from subgroup IVa and IVb showed a deviation of 12-20% between their expected and observed size. The deviation of fusion proteins from expected molecular mass on SDS-PAGE gels have been reported previously (Jain, 2013; Liang *et al*., 2012; Rath *et al.,* 2009; Shi *et al*., 2012a; Wang *et al.,* 2013). Several factors can influence aberrant migration of polypeptides on SDS-PAGE gels. Major factors include, primary amino acid sequence (Bjellqvist *et al*., 1995), post translational modification (Georgieva & Sendra, 1999), secondary structures (Saha *et al.,* 2009) or biochemical interaction between amino acids and SDS resulting in change in the net charge of the polypeptide chains (de Jong *et al*., 1978).

The SDS reacts with protein molecules at their hydrophobic site helping the protein to form an α-helix and randomly coiled structure, a phenomenon termed as "Reconstructive Denaturation" (Imamura, 2006). SDS with the random coils form a "necklace and bead structure" (Shirahama *et al*., 1974) where the size of the beads can vary depending on the amino acid sequence (Ibel *et al*., 1990). Lower net positive charge or higher net negative charge of the protein and presence of aromatic amino acids promote the binding of SDS to the proteins, thus favoring their migration in a PAGE gel (Shi *et al*., 2012a). Among the recombinant CBFs examined during this study, both TrxHisS-CBF9 isoforms had lower net positive charge and almost 6-7% aromatic amino acids in their primary sequence (Table 4.7). This might have favored better migration of these TrxHisS-CBFs and resulted in almost identical expected and observed molecular weights (Table 4.3). Thus, even though sequence wise these two CBFs belong to two different lines (Fig. 4.14), functionally they showed similarity. Previous study with TrxHisS-CBFs has shown effectively that either the AP2 domain or the N-terminal region might be responsible for the slow migration (Jain, 2013). But, this could not explain the faster migration of some of the TrxHisS-CBFs (sub-group IVd).

The presence of proline residues provides a kink in the protein secondary structure, which does not straighten when denatured (Nelson *et al*., 2005). This results in a larger radius of the

protein to travel through the polyacrylamide gel instead of the linear form and leads to slow migration of the proteins during PAGE. The TrxHisS-CBF9 and -CBF22 isoforms have a proline content that ranged from 5.9- 6.2%, whereas, the slowest migrating TrxHisS-CBFs from group IIId have a proline content of 7.8-9.4% (Table 4.7). Overall, it can be said that the N-terminal coding region and AP2 domain along with the percentage of proline residues present in the primary amino acid structure, might have together resulted the slow migration of the recombinant CBFs.

Post translational modifications, especially glycosylation was also reported for slower migration of proteins during SDS-PAGE analyses (Tanaka *et al*., 2011). Glycosylation also reduces the binding of Coomassiae blue to the proteins and thus gives a diffused appearance of the protein bands (Gao *et al*., 2001; Luzio *et al.,* 1990). In this study, no such anomaly was observed during staining and hence, glycosylation can be ruled out as one of the reason for the anomalous migration of the TrxHisS-CBFs on gels.

5.4. Preliminary results suggest structural variations exist in the CBFs studied

CBFs evolved as a defense mechanism in ancestor wheat species during the climatic transition from warmer to colder conditions. With the development of polyploidy, wheat developed increased copy numbers of CBF genes. It may be that not all these CBFs are functionally active or have similar activities. All the fifteen TrxHisS-CBF purified in this study were stored at -80°C with 10% v/v glycerol as a cryo-protectant. Repeated freeze and thaw cycles did not affect the migration of the CBF fusion proteins on SDS-PAGE, or showed the presence of degradation products. The TrxHisS-CBFs also retained their DNA binding ability even after three freeze-thaw cycles. During this project, three TrxHisS-CBF2 isoforms, two TrxHisS-CBF9 isoforms and one each of TrxHisS-CBFIIId-B12, -CBFIIId-A15, -CBFIVb-B20 and -CBFIVb-D21 were tested for their DNA binding ability.

Subtle amino acid differences might be responsible for the activation-inactivation of DREB1/CBF transcription factors (Zhao *et al*., 2007). There is one amino acid change from Proline to Arginine in the amino terminal CMIII-3 sequence that could reduce the binding affinity of CBFIVa-2.3 than the other two CBF2 isoforms. Other than this, very few amino acid residue changes were observed (Q9-H9; Q23-nil) at the amino-terminal coding region sequence

of the CBF2 isoforms. However, there are several changes towards the carboxy-terminal end and future studies might be required to decipher the role of the C-terminal in determining the affinity towards CRT motif. One of the several factors affecting stability of proteins is presence of four amino acid residues- Proline (P), Glutamic acid (E), Serine (S) and Threonine (T), commonly called PEST residue (Rechsteiner & Rogers, 1996). When the PEST scores for all the three CBF2 isoforms were calculated using available online tools, they ranked as following: CBFIVa-A2 < -CBFIVa-A2 like < -CBFIVa-2.3 (Table 4.4). The DNA binding ability of these CBFs as TrxHisS-fusion proteins might have reflected the same trend, where instability of these CBFs was inversely proportional to their DNA binding ability. The most potent DNA binder at 4°C was TrxHisS-CBFIVa-A2 which had the lowest instability due to low PEST score, followed by TrxHisS-CBFIVa-A2 like and -CBFIVa-2.3 respectively (Fig. 4.16; Table 4.7). In summary, the CBF present on group 5 chromosomes show subtle differences in their structure, that supports the hypothesis for this work.

5.5. Purified TrxHisS-CBF fusion proteins retained their functional activity

When the functionality of the TrxHisS-CBFs after purification was tested, the preliminary data revealed that most of these recombinant CBFs showed variation in their DNA binding activity. Most of them showed increased affinity to the CRT motif when subjected to incubating at a lower temperature. Only exception was TrxHisS-CBFIIId-B12, which showed almost identical binding intensity at RT and 4°C (Fig. 4.18B). TrxHisS-CBF12 isoforms from winter wheat have previously been shown to not to be affected by the temperature in terms of their DNA binding ability (Jain, 2013). Data from this study too showed a similar trend followed by the TrxHisS-CBFIIId-B12 obtained from *Fr-D2*. Earlier it was also reported that *in vitro* DNA binding ability of barley *Hv*CBF2 varies based on the incubation temperature where it showed ten times higher binding when incubated at 0°C compared to 25°C (Xue, 2003). Preliminary data obtained from this study showed that the same trend was followed by CBF2 isoforms obtained from winter wheat *cv.* Norstar D genome. TrxHisS-CBFIIId-B15 showed DNA binding ability but surprisingly, in this study TrxHisS-CBFIIId-A15 could not bind to the CRT motif (Fig. 4.16, 4.17). There have been reports of DREB1/CBF transcription factors that remain inactive during the early stages of acclimation only to become active during the later

stage to stop the process in a competitive manner (Zhao *et al*., 2006). It might be possible that CBF15 isoforms function in antagonistic way *in planta*.

CBFs from winter wheat can be differentially expressed based on time and duration of cold acclimation period (Kume *et al*., 2005). In Arabidopsis too the expression of CBF cascades is considered as an early event during cold acclimation (Gilmour *et al.*, 1998). Even though the *in vitro* experimental conditions do not necessarily reflect the *in vivo* conditions, but it can be assumed from the comparative study that CBFs from subgroup IIId and IVa might be effective for a longer period and a wider temperature range during cold acclimation process.

The 15 recombinant CBFs were purified following the methods optimized in objective 1. The EMSA assays showed that the majority of the TrxHisS-CBF could retain their functionality as DNA binding proteins. A few TrxHisS-CBFs did not bind, irrespective of the various physical conditions used during extraction, purification or EMSA assays, identical to the DNA binders. Thus, it cannot be concluded that these non-binding proteins were either pseudo-proteins or became functionally inactive due to the purification procedure. Rather, based on the active DNA binding properties of the majority of these recombinant CBFs, it may be considered that physical conditions were not conducive for the non-binding of these TrxHisS-CBFs and that these CBFs were not good binders themselves.

5.6. Conclusion

In summary, an optimized method to produce recombinant TrxHisS-CBF has been developed. The method involved physical disruption of bacterial cells to release recombinant CBF in to cell soluble extracts. An automated FPLC based affinity chromatography, followed by ion exchange chromatography purified the recombinant TrxHisS-CBFs to apparent homogeneity and in adequate quantities for functional analyses. DNA sequence analyses followed by predicted peptide structures and bioinformatics analyses revealed structural differences among CBFs and also within isoforms of the same CBF such as CBF2. This proved the hypothesis that structural differences exist between different CBFs. Functional analyses using EMSA and SPR confirmed that TrxHisS-CBF fusion proteins extracted and purified following the standardized protocol developed during this study can be suitably used for functional studies.

5.7. Future studies

Variations in the CBF structure and their DNA binding ability was observed in CBFs, belonging to the same or closely related subfamily. The source of the CBFs was Norstar, which is one of the most cold-hardy winter wheat varieties in Canada, with an LT_{50} value of -27°C. Wheat is an allohexaploid, making it a complex genome with three copies of gene each derived from a different progenitor. Subtle differences were observed in the amino terminal end of CBF, however, the role of carboxy-terminal in determining DNA binding ability of the AP2 domain needs to be studied. The implications of subtle amino acid substitution/changes observed in this study in frost tolerance in wheat also needs further investigation. Site directed mutagenesis or deletion studies could help to understand the structure-function relationship of the CBFs. Similar studies on better cold tolerant winter rye and Nordic grasses should be performed to obtain more insight on the role of CBFs to determine cold tolerance and winter field survival.

CHAPTER 6

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CHAPTER 7

APPENDICES

Table 7.1. List of primers used for PCR amplification of CBF genes.

CBF	Forward primer	Reverse primer
CBF IVa-A2 like	GACGACGACAAGATGGACACGTCGGCGC	GAGGAGAAGCCCGGTCTAGTAGCTCCACAGCGACGTGTC
CBF IVa-2.3	GACGACGACAAGATGGACACCATGGCCGC	GAGGAGAAGCCCGGTCTAGTAGCTCCACAGCGACGTGTC
CBFIVa-A2	GACGACGACAAGATGGACACCGTTGCCGC	GAGGAGAAGCCCGGTCTAGTAGCTCCACAGCCACGTGTCGAA
CBFIIId-B12	GACGACGACAAGATGGACACGGGCCCC	GAGGAGAAGCCCGGTTAGTGGTTCCATAGCGCCG
CBFIVd-B9	GACGACGACAAGATGGACGTCGCCGACATC	GAGGAGAAGCCCGGTTAGTCGAACAAGCAGCTCCATATG
CBFIVd-D9	GACGACGACAAGATGGACGTCGCCGACATC	GAGGAGAAGCCCGGTTAGTCGAGCAAGCAGCTCCATAG
CBFIIId-A15	GACGACGACAAGATGGACATGACCGGCTCC	TAGTGGTTCCATAGCGCCGTAGTAGCTCCAGAGCGCGG
CBFIIId-D19	GACGACGACAAGATGGACACGGCCATCGA	TAGTGGTTCCATAGCGCCGTAGTAGCTCCAGAGGCCGGC
CBFIVb-B20	GACGACGACAAGATGGACACCGCCGCTG	TAGTGGTTCCATAGCGCCGTAGTTCCAAAGCGGCGTGTAGA
CBFIVd-B22 like	GACGACGACAAGATGGACGTCGCCGACG	TAGTGGTTCCATAGCGCCGTAGTTGAACAAGTGGCTCTGGCTC
CBFIVb-D21	GACGACGACAAGATGGACGCCGACGC	TAGTGGTTCCATAGCGCCGTACCATAGCGGCGTGTAGA
CBFIIId-A12	GACGACGACAAGATGCACACGGCCCCC	GAGGAGAAGCCCGGTTAGTGGTTCCATAGCGCCG
CBFIVd-B22	GACGACGACAAGATGGACGTCGCCGACG	TAGTGGTTCCATAGCGCCGTACACTGCTAGATTAGGTGAACAAGTGGC

CBF names are based on Badawi *et al*., 2007.

Table 7.2. List of Primer sequences used for EMSA and SPR assays

Primer name	Sequence
Wcs120 F	5′-GCCACCTGCCGACCACTGATC-3′[¥]
Wcs120 R	5′-GATCAGTGGTCGGCAGGTGGC-3′

[¥] CRT motif is underlined

Table 7.3. TrxHisS-CBF/CRT interaction data obtained from SPR study

CBF isoforms	K_a	K_d	K_D
IIId-B12	$9.64E^{+04}$ 1/Ms	$2.60E^{-02}$ 1/s	$26.9E^{-06}$ M
IIId-A12	$1.11E^{+04}$ 1/Ms	$3.47E^{-02}$ 1/s	$3.13E^{-06}$ M

K_a; Association rate, K_d; Dissociation rate, K_D; Affinity of the reaction

Table 7.4. List of *E. coli* cell types used during this study

E. coli	Used for	Genotype
Novablue Gigasingles	Plasmid amplification	*endA1 hsdR17*(r_{K12}-m_{K12}+) *supE44 thi-1 recA1 gyrA96 relA1* lac F′[*proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*::Tn*10* (Tc^R)]
BLR(DE3)pLysS	Protein over-production	F$^-$ *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm lac tle* (DE3) Δ (*srl-recA*)306::Tn*10* pLysS (Cam^R, Tet^R)

Fig. 7.1 Multiple sequence allignment of the CBFs purified during this study using CLUSTAL 2.1 online available tool (Larkin *et al*., 2007). The CMIII-1 and CMIII-3 conserved flanking sequences are colored red and italicized. The amino acids conserved among all the CBFs are marked by (*). CBF names are based on Badawi *et al*., 2007.